THE METABOLISM OF PLASMA PROTEINS IN THE YOUNG CALF

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by

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SECTION I

GENERAL INTRODUCTION

The development of our knowledge about the plasma proteins has been very dependent on the techniques available at any one time. Part of this development has been a growing awareness of the importance and complexity of antibodies. Landsteiner, working from 1906 to 1943 showed that the phenomena of immunity are essentially chemical, antibodies being adapted or slightly modified serum globulins. He established the concept of immune specificity.

The first techniques applied to the investigation of plasma proteins depended on their solubility and related properties. Tiselius (1937) introduced electrophoresis which along with the development of the ultracentrifuge by Svedberg, has proved invaluable. Cohn's fractionation with ethanol at low temperatures provided a method for the production of large quantities of individual plasma proteins for further studies.

In his review, Wormall (1948) emphasised that in the future, more modern weapons would be used to tackle immunochemistry, in particular, the use of isotopic tracers. Prior to this, it had been possible to study plasma protein metabolism using balance studies and infusions (Jarnum, 1965). Thus, Welch, Adams and Wakefield (1937) observed a high faecal nitrogen output in a case of severe ulcerative colitis. Such studies were however very limited. Isotopically labelled proteins proved to be a major advance.

Early isotopic studies were complicated by problems associated with the purity of plasma protein fractions and with the risk of denaturation both before and after labelling. This was emphasised by Kekwick (1966) who pointed out how labile some purified plasma proteins were, the avoidance of damage during separation requiring unremitting vigilance.

The development of the technique of Immunoelectrophoresis by Grabar and Williams (1953) demonstrated how heterogenous many of the plasma protein fractions were, in particular gamma globulin, as previously defined by electrophoresis.

The improved separation techniques of molecular sieve chromatography and ion-exchange chromatography, developed in the 1950's, have overcome many of the problems associated with earlier techniques. Finally, the development of an in vitro method of isotopic labelling (McFarlane, 1958) which did not alter the metabolism of the protein under investigation made possible the very extensive study of plasma protein metabolism, which has subsequently taken place. This investigation is an application of these improved separation and labelling techniques to a study of the metabolism of plasma proteins in the young calf.

The plasma proteins of the new-born calf can be divided into those present in the foetus and therefore at birth, and

those absorbed from the alimentary tract following the ingestion of colostrum. However, this is not a rigid subdivision as the foetus may produce antibodies under certain conditions. It may be further or similarly complicated in the weeks following birth by the developing capacity of the calf to synthesize its own antibodies. Both these possibilities will be discussed. Even so the main change in the serum proteins of the neonatal calf is the acquisition of maternal antibodies from the colostrum. The lack of such protection has been linked with the high mortality among new-born calves of which Colibacillosis (see Section VII - Discussion) is probably the major cause.

Jordan (1933) noted a 40% mortality, which virtually disappeared at pasture. Withers (1953) reported the results from 44 herds throughout Britain. On individual farms he found that the death rate might exceed 25% although the overall mortality was 8.3%. This was among heifer calves, up to 6 months old, in dairy herds. Hagan (1956) reported 20% losses of calves from a common enteric ailment. Ottosen (1959) in a postmortem survey concluded that 43% of the calves examined had died from Colibacillosis. Gay, Anderson, Fisher and McEwan (1965) reported a 17.4% mortality among market bull calves, in the West of Scotland.

More recently Sellers, Smith and Wood (1968) found a 21%

mortality in the first week of life, with an additional 4% in the second week. de la Fuente (1970) in a farm survey carried out in the West of Scotland, showed that the highest incidence of death from neonatal diarrhoea occurred from December to March, coinciding with the greatest number of hypogammaglobulinaemic calves. Other reviews which associate a high proportion of neonatal calf mortality with Colibacillosis include those of Lovell (1955), Penhale (1965), Sojka (1965), McEwan (1968) and Roy (1970).

The investigation will therefore concentrate on those plasma proteins acquired by the calf during the first few hours of life. Since Ehlrich (1892) demonstrated the transfer of colostral antibodies in mice, the study of this acquired immunity has been tied up with the investigations of antibodies as a whole.

Landsteiner and van de Scheer (1936) demonstrated the production of antibodies of varying specificity. Kabat and Pedersen (1938) using the ultracentrifuge found two groups of antibodies with very different sedimentation coefficients, 6.75 and 18.75 respectively. Tiselius and Kabat (1939) confirmed the species difference found by Kabat and Pedersen. Cohn, Deutsch and Wetter (1950) reported the existence of an additional group of antibodies, sedimenting between 8s and 12s.

Cann (1953) examined antibodies of the same sedimentation value and found a whole range of electrophoretic mobilities and isoelectric points. Franklin and Kunkel (1957) showed that the 19s fraction was a constant constituent of normal γ globulin and antigenically related to the major 7s component. Raynaud (1959) demonstrated the marked heterogeneity of diptheria antitoxin, produced in horses.

The advent of much improved separation techniques led to a considerable increase in our knowledge of this heterogeneity. Fahey and Horbett (1959) separated 19s and 7s components by ion-exchange chromatography and found differences in electrophoretic behaviour, hexose content and antibody distribution, although the components were obviously antigenically related. Speer, Prager, Kelley and Hill (1959) and Porter (1960) arrived at similar conclusions. Talmage (1959) on the other hand emphasised the indivisibility of antibodies, because of their diversity.

Eisen and Siskind (1964) continued the much earlier work of Landsteiner. Their findings were similar to those of Uhr (1964) in his investigation of the multicomponent immune system. Kabat (1966 a and b) and (1967) emphasised this heterogeneity, which Kunkel (1967) found staggering, with such a large array of classes and subclasses, forming a heterogeneity above and beyond that directly involved in antibody specificity. He estimated that an individual can form about 50,000 different antibodies.

By immunoelectrophoresis Heremans (1959) and (1960) found a population of similar but electrophoretically heterogeneous proteins, in the β_2 and γ regions all called " γ globulins ", for which he suggested the term immunoglobulin. Schultze, (1959) put forward a similar concept. Kabat and Mayer (1961) found that although one of these, γ_G immunoglobulin (three had so far been discovered) spread over a considerable range on electrophoresis, antigenically it was one protein. Franklin (1962), Kunkel (1962) and Fahey and McLaughlin (1963) all investigated and commented on the electrophoretic heterogeneity of the immunoglobulins. Fahey and McLaughlin demonstrated class specific and common antigenic determinants, showing that they could be defined on immunochemical grounds. Working with myeloma proteins, Mannik and Kunkel (1963) discovered the presence of two antigenically distinct groups of γ_G in man.

Graber (1963) tried to tidy up the confused nomenclature but this was finally achieved by the World Health Organisation (1964). The classes were named using the abbreviation Ig or the symbol γ thus, IgG or γ_G, IgA or γ_A, IgM or γ_M etc. Fudenberg (1965) stated that each of these groups contains an indeterminate number of proteins which share certain structural, physico-chemical and antigenic properties and at the same time certain specific individual properties. Kabat (1966b) and Burnet (1969)

considered that it had been the development of Immunoelectrophoresis which had so increased our insight into these heterogenous antibodies.

Subsequent research into the basic structure of the immunoglobulins was greatly facilitated by the availability of myeloma and Bence-Jones proteins. Kunkel, Killander and Mannik (1966) found that all the evidence was in favour of the close relationship between these proteins and antibodies. Furthermore, cold agglutinin and rheumatoid activity had been demonstrated in some Waldenström macroglobulins. Kabat (1967), Webb and Goodman (1967), Fahey (1968) and Rowe (1968) are but a few of the workers who have investigated and discussed the heterogeneity of antibodies from every angle, including the structural one.

A similar complexity has been demonstrated in bovine serum. The initial differentiation between the two γ_G components depended largely on electrophoresis, with its' limitations. Thus Smith (1948) showed that the colostral immune globulins were closely related to the γ and I serum globulins. Subsequently, Smith and Holm (1948) found no change in mobility after absorption of colostral immune globulin by the calf. Hess and Deutsch (1948), using a modified cold ethanol fractionation proceedure, prepared two closely related γ globulin fractions from bovine serum.

Other workers used their own nomenclature. Thus Polson (1952) refers to γ_1 and γ_2 globulins, Pierce (1955) to γ_1, γ_2 and γ_3 globulins, Larson (1958) to β_2 and γ_1 globulins and Porter and Press (1957) to part of bovine γ globulin closely related to the β globulin. The main conclusion from all this work, concerning the presence of two closely related γ globulin fractions with differing electrophoretic mobility, has subsequently been fully substantiated.

Carrol (1961) showed that γ components varied as to the conditions under which they were eluted in ion-exchange chromatography. Pierce (1961) confirmed the identity of colostral immune globulin with T or γ_1 component of serum. Murphy, Aalund, Osebold and Carrol (1964) and Murphy, Osebold and Aalund (1965) described slow and fast 7s components in bovine lacteal secretions, the relative amounts of which varied depending on the character of the secretion. i.e. colostrum showed an almost complete absence of the slow component. They considered this all the more remarkable, in the light of the similarities between the two proteins, which differed only as regards net electric charge, a minor sedimentation coefficient difference, a minor elution position variation when F fragments were chromatographed and the association of complement fixation with the fast fraction. The antigenic difference between them was associated with the spur formation obtained on Immunoelectrophoresis.

Aalund et al (1964) refered to the Fast and Slow IgG of the guinea pig where the two fractions can be defined on their capacity to mediate particular biological functions (see Benacerraf, Ovary, Block and Franklin, 1963, and Nussenzweig and Benacerraf, 1967). Mansa (1965) refers to the fast component as γ_1 A globulin.

Pierce and Feinstein (1965) carried out further studies into the relationship between colostral and serum immunoglobulins. Subsequently Pierce (1966) designated the slow and fast fractions as sub-species IgG_1 and IgG_2. Cohen and Milstein (1967) in their review of the immunoglobulins state that it is the Fc portions of the respective heavy chains which account for the slight antigenic difference between γG_1 and γG_2. Additional evidence of the differences and similarities between them was produced by Milstein and Feinstein (1968).

Aalund (1968) showed that the bovine IgGs cross reacted with human IgG. He found the same hexose content and plasma $T\frac{1}{2}$ for colostral and serum Fast IgG. Kickhofen, Hammer and Scheel (1968) found some evidence of slight alteration during absorption of colostral γ_G which they differentiated from serum γ_1 and γ_2.

Butler (1969) emphasised the heterogeneous nature of both bovine IgGs, compared to other species. He suggests that the two subclasses may be an over simplification.

Rice and Carriere (1969) found relatively high complement fixing titres with a Mycobacterium johnei polysaccharide in post-colostral calf sera containing little or no slow IgG. Sullivan, Prendergast, Antines, Silverstein and Tomasi (1969) found that fast IgG was also the principal bovine immunoglobulin in saliva and tears, in addition to colostrum. Nansen (1970) showed that the metabolism of IgG_2 was similar to that of other mammalian IgGs, whereas fast IgG appeared to deviate from the typical pattern. Other workers, Brindley Morgan (1967), Klaus and Jones (1968), and Stone and Gitter (1969) have studied bovine IgG, without reference to the two subclasses.

Johnson and Pierce (1959) identified a small proportion of macroglobulin (18s) in colostral whey. Kramer (1963) detected maximal E.Coli agglutinin activity in the β_2 region and purified this fraction. Murphy et al (1964a) noted the presence of γ - 1M immunoglobulin in bovine lacteal secretions. The same group of workers, (Murphy, Aalund and Osebold, 1964b) demonstrated and investigated the heterogeneity of this protein with which they associated complement fixing activity. They also investigated its' physico - chemical properties, (Murphy, Osebold and Aalund, 1965) identifying it on the basis that it produced an arc on immunoelectrophoresis identical to that of human γ M. Jenness, Anderson and Gough (1965) and Gough, Jenness

and Anderson (1966) reported finding Brucella agglutinins associated with the IgM fraction.

Pierce (1966) and (1967) supported the earlier evidence for the presence of a 19s macroglobulin in bovine sera. Brindley Morgan (1967) indicated the importance of IgM antibodies to Brucella. Hammer, Kickhofen and Hemming (1968) in their studies of primary and secondary responses to antigens in cattle, differentiated between 19s γ globulin and the γM component. They compared γM and γG antibodies. Klaus and Jones (1968) found raised IgM levels in experimental Anaplasma marginale infections in calves. The same workers (Klaus, Bennet and Jones, 1969) investigated the absorption of IgM and IgG in the new-born calf. Using the same technique Penhale and Christie (1969) quantitated these immunoglobulins in adult plasma and colostrum and subsequently in neonatal calves (Penhale, Christie, McEwan, Fisher and Selman, 1970). Rice and Carriere (1969) identified low levels of IgM in the serum and colostrum of cows with non-clinical Johnes Disease. According to Butler (1969) the antigenic distinctive component of Bovine IgM appears to reside in the Fc fragment which shares no antigenic determinants with bovine IgG. Complement fixation studies have shown that bovine μ- chains cross react with anti - human μ - chains.

It was originally thought that calves were born completely

without γ globulin and that the foetus was unable to produce antibodies. Hansen and Phillips (1947) and Pierce (1955) detected very low levels of γ globulin in pre-colostral serum of new - born calves. Fennestad and Borg - Petersen (1957) found evidence that the bovine foetus can produce antibodies against Leptospira saxkoebing. Subsequently the same workers (Fennestad and Borg - Petersen, 1962) experimentally infected foetuses and subsequently detected antibodies in them. Schmid and Buschmann (1962) were unable to detect blood group antibodies in the 311 foetal sera which they examined. As a result of the development of special highly specific typing systems for genetic γ globulin factors, Martensson and Fudenberg (1965) were able to demonstrate that the human foetus may be able to synthesise small amounts of IgG. Kniazeff, Runner and Gaeta (1967) found significant amounts of γ globulin in about 40% of foetal sera examined. There is thus considerable evidence that the foetus can produce antibodies thus explaining the low levels of immunoglobulin detected in foetal and pre-colostral calf sera by many workers.

Other workers have investigated the bactericidal activity of pre-natal and post natal calf sera. Turk (1959) suggested that this activity was due to the interaction of properdin and complement. Muschel (1960) however considered that there was insufficient evidence for this, while Osawa and Muschel (1960)

concluded that in the presence of antibody, properdin might augment the bactericidal titre to a slight degree. Penhale (1965) investigated the pre-colostral serum factor (P.S.F.). He considered it to be similar to properdin and distinct from IgM and IgG. He demonstrated its' bactericidal properties. More recently, Collins, Carroll, Jasper and Jain (1970) confirmed the bactericidal properties of foetal and pre-colostral sera. It will therefore be necessary when estimating changes associated with the absorption of colostral antibodies to determine the level of any pre-colostral immunoglobulin. P.S.F. is distinct from this and in relation to the protective role of the colostral immunoglobulins, probably not quantitatively important.

Evidence of the ability of neonatal animals in general and the calf in particular to respond to antigenic stimuli is available from a number of sources. Kerr and Robertson (1954) using Trichomonas foetus were unable to detect antibodies before one month and Kerr (1956) found that five out of six calves did not respond to Brucella or Salmonella antigens. Penhale (1965) detected antibodies against specific E.Coli strains, 28 to 36 days post innoculation, in five out of six calves. In three hypogammaglobulinaemic calves, antibodies were present by one month. More rapid responses have been found. Thus Brown (1956) using a viable Rinderpest lapinsed virus detected antibodies at 21 days and in children, Smith (1960), showed

that infants given Salmonella antigens at birth developed high titres at 7 to 14 days. Smith and Ingram (1965) concluded that immunological competence occurs at different ages, depending on the character of the antigens. There is obviously a species variation as well.

The inhibitory effect on the production of antibodies by passively acquired maternal antibodies has been demonstrated and commented upon by a number of workers. Brown (1958) showed that calves born to Rinderpest immune cows did not respond to vaccination under 3 months. Schrieier and Porath (1964) noted the strong inhibition of synthesis in young rabbits. Rowley and Fitch (1964) found that passive immunisation inhibited the primary response in rats. This may be a related phenomenon. The inhibitory effect of colostral antibodies was discussed by Pierce (1961) and Smith and Ingram (1965). Another approach has been to investigate γ globulin levels. In children the lowest γ globulin levels are found at 2 to 3 months (Ulstrom, Smith and Heimlick, 1956, and Putman, 1960). The initial fall and subsequent rise were clearly demonstrated by Gitlin and Janeway (1957) and confirmed by Hobbs (1966). Bridges, Condie, Zak and Good (1959) correlated this initial fall with the absence of plasma cells and the failure to form them. They found considerable individual variation in the appearance of

plasma cells in the gut, from 3 weeks up to 3 or 4 months. This was reflected in the variation found in the period of γ globulin decline. The need for external antigenic stimuli in the development of normal γ globulin levels has been shown for the chicken, rat and the mouse, using germ free stock (Borsos and Kent, 1958, Gustafsson and Laurell, 1958 and Sell and Fahey, 1964). In calves deprived of colostrum, Pierce (1961) found electrophoretic evidence of the synthesis of 7s - γ globulin by the end of the first week of life.

Thus although neonatal animals in general and calves in particular can produce antibodies to certain antigens within the first month of life, synthesis of γ_G does not reach adult levels till later. Hansen and Phillips (1947) showed that in colostrum deprived calves, normal γ globulin levels were not approached till 8 weeks. This is in animals which have not been under the inhibitory influence of passively acquired colos tral antibodies. Thus for the purpose of these studies which will be largely confined to the first 2 or 3 weeks of life, the role of synthesis can be ignored.

The metabolism of Fast IgG and IgM will therefore be investigated with a view to quantitating the efficiency of absorption from colostrum of both these immunoglobulins and assessing their role in relation to the alimentary form of Colibac illosis.

SECTION II

GENERAL MATERIALS AND METHODS

A. EXPERIMENTAL ANIMALS

Ayrshire calves (mainly bull calves) were used throughout the experiments. They were obtained from different sources, details of which are given in the individual sections.

1. Housing

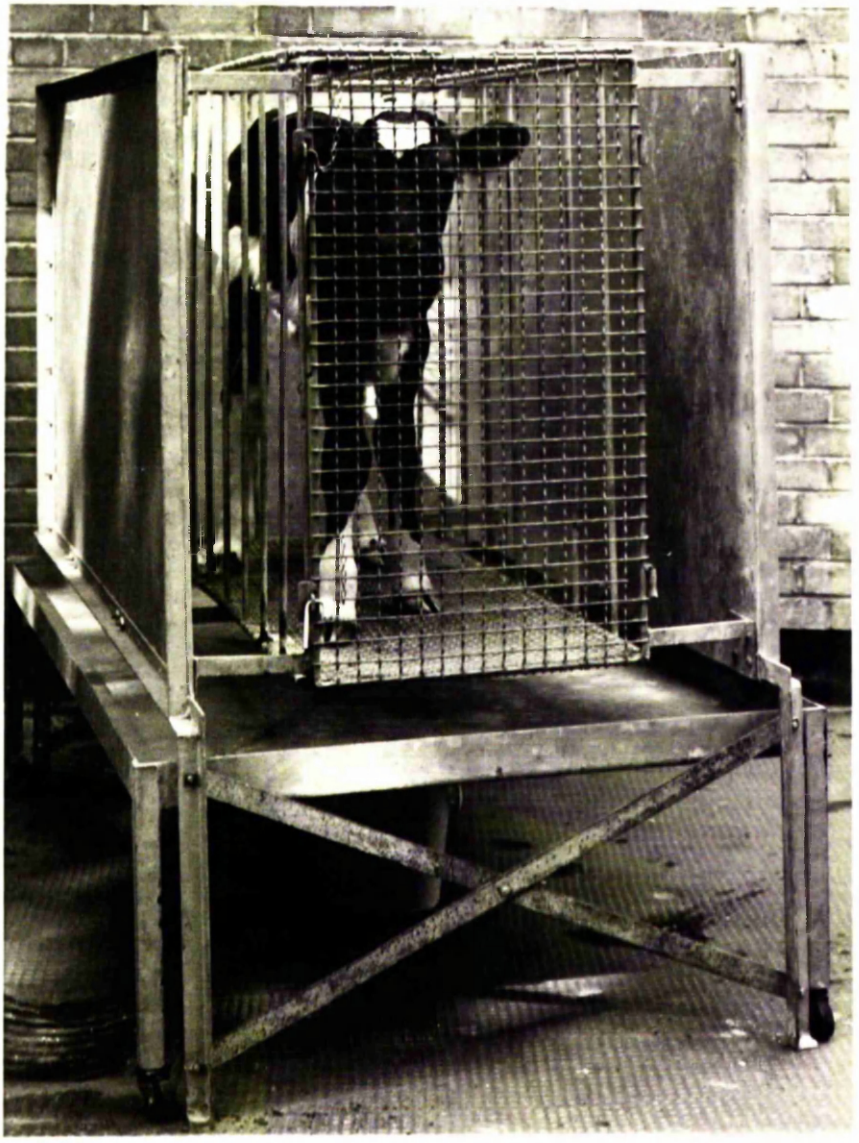
The calves were kept in metabolic cages (see Section B) so that urine and faeces could be collected. It was not possible to keep the environmental temperature constant as calves were housed under a variety of conditions depending on the accommodation available. Calves which were removed from the dam at birth were provided with supplementary heating from an infrared lamp for the first 17 days of life. Similar lamps were provided for any calves which were shivering or appeared dull. When not in metabolism cages, the calves were housed in concrete pens with oat straw bedding. The cages and pens were thoroughly cleaned and disinfected ('White Septol B', Robert Young & Co., Glasgow) between calves.

2. Feeding

The calves were fed twice a day with warmed whole milk, consuming up to six pints at each feed depending on appetite. Where calves developed diarrhoea, no change was made in the feeding pattern, but frequently, the calves reduced their intake.

Figure 1

Calf in a Metabolism Cage (II A 1)



In the isotopic studies all calves received 1.3 gms of KI/day/30Kg body weight prior to and during the experimental period. The Iodide solution was given by mouth.

When the calves were kept beyond the age of one month (C 9 and C 13 in Sections IV, V and VII, and C 18 in Section V) hay and calf weaner pellets (W. Primrose and Sons, Glasgow) were gradually introduced. The final diet consisted of ad lib hay and water and approximately 3lb/day of pelleted concentrate.

B. METABOLISM CAGES

Two types of cage were used.

I. Galvanised Metal Cages

Cages were required which would effectively confine the calves, allow easy access and ensure that all urine and faeces could be collected. In anticipation of the particular problems of collecting faeces from diarrhoeic calves, it was decided to have cages built to specification. (Construction was undertaken by C.F. Howden Ltd., Glasgow).

The final design (see Fig. 1) consisted of an inner and outer chamber. The dimensions of the inner chamber were such that the calf was discouraged from turning round. It had three walls of 3/8 inch vertical rods at 6 inch intervals, a door, at the front covered by ½" wire mesh and a ½" metal grid as base. The outer chamber had three sides of solid metal sheeting. The top of

the cage was partly enclosed by two hinged ½" wire mesh flaps 7" wide, running the full length of the cage. Each cage had a pair of moveable collecting trays. All excreted material produced by the calf was collected in a bucket placed under the tray. The tray and cage could be washed and brushed down to achieve a complete collection.

2. Tubular Cages

These cages were used in experiments when more than two cages were required or when it was necessary to separate urine and faeces.

The framework was made from 1" galvanised tubing joined together with "Kee Klamps" both supplied by Gascoignes Ltd., Glasgow.

It consisted of a main outer frame which formed the support for the calf grid and for the adjustable inner cage. It also provided support for a sloping grooved aluminium sheet positioned under the inner cage, for the collection of urine. The inner cage could be adjusted for width and length as required. The galvanised metal grid, with a rectangular mesh 1" by 2" was made by W.M.Reid & Sons, Glasgow.

These cages were used in two different ways:-

a. as a substitute for the solid galvanised cages, by enclosing the cage in 500 guage polythene sheeting (Transatlantic

Plastics Ltd., Ventor, Isle of Wight) attached to the sheet of aluminium and the outer frame in such a way that the only access to the calf was from the front of the cage. Additional access was provided on one side by making a flap in the polythene. A reinforced hole was then made at the lowest point in the "polythene cage", where the polythene was attached to the aluminium sheet. All excreted material could then be collected.

b. by using the faecal collection method developed by de la Fuente (1970). The urine was allowed to run down the aluminium sheet into a receptacle. The faeces were collected in long polythene bags attached to the rear end of the calf by means of a brieket harness and tight fitting "rubber chute" adapted from gloves designed for the rectal examination of cattle. For further details see de la Fuente (1970).

C. ISOTOPE TECHNIQUES

1. Isotopically Labelled Plasma Proteins

a. Labelling

The iodine monochloride method of McFarlane (1958) as described by Dargie (1969) was used throughout.

(i) Iodine Monochloride

The stock solution was made up according to the method of Vogel (1951). For labelling, a 1 in 330 dilution in saline was freshly prepared.

(ii) Buffer Solutions

100ml of M/1 Glycine in M/4 saline was prepared. Buffer A was made up by adding N/1 NaOH to 45ml of the glycine solution till the pH was 8.5. Buffer B was similarly prepared by adding N/1 NaOH to 40ml of the glycine, till the pH was 9.0.

(iii) Protein for Labelling

A 2% solution of the protein was buffered with Buffer B (1ml of buffer to every 2ml of protein).

(iv) The Isotope

$Na^{125}I$ or $Na^{131}I$ was obtained from the Radiochemical Centre, Amersham, free from reducing agent (i.e. thiosulphate free). Prior to labelling the volume was increased from 0.1ml to 5ml with saline.

(v) Iodination

The isotope was rapidly pipetted into a predetermined volume of Iodine Monochloride (such that the number of atoms per molecule was kept at the desired level), mixed and then buffered with Buffer A (the same volume as Buffer B). The solution was then rapidly pipetted into the buffered protein.

(vi) Dialysis

After thorough mixing, the labelled protein was carefully poured into a dialysis sac containing enough bovine albumin to reduce the specific activity of the final

preparation to less than 5μc/mg. The protein was then dialysed in 25 - 30 litres of isotonic saline, for 48 hours, with one change of saline.

(vii) Protein Bound Activity

Before injection or storage at -20°C, the percentage (bound activity) was determined in 0.1ml of the labelled protein. It was precipitated with 4.9ml of 10% Trichloracetic acid and after standing for 30 minutes at 4°C was centrifuged. The precipate was suspended in 5ml of dilute NaOH in a counting tube after the supernatant had been transfered to another tube. Both tubes were then counted (see Section II C 7) and the percentage bound activity calculated. By counting samples of the saline used for dialysis, an estimate was also obtained of the labelling efficiency.

b. Injection

Weighed syringes were filled with the labelled preparation one syringe for each calf, and one for a radioactive standard. The protein was then injected via a jugular catheter (Portland Plastics Ltd., Hythe, Kent) previously introduced under local anaesthesia. After injection, the catheter was flushed with saline. The empty syringe was later reweighed.

When it was intended to carry out repeat Plasma Volume determinations, the catheter was secured to the neck of the

calf with 2" elastoplast (one piece forming a panel round the top of the catheter and a second one holding the panel in position, by going right round the calf 's neck). The catheter was closed with a nylon stilette, (1mm diameter) which could be subsequently withdrawn. During the period that the catheter was left in position, antibiotic cover was provided. The calves were given daily intramuscular injections of 600,000 units/30Kg of Procaine Penicillen G ('Mylipen', Glaxo, Greenford) and 1gm/30Kg of Streptomycin/Dihydrostreptonycin ('Dimycin', Glaxo, Greenford)

2. Oral Administration of Radioiodinated Fast IgG

Method of preparation, as above (Section II C 1). For further details of administration see Section V.

3. Injection of Other Isotopically Labelled Compounds

a. Radioiodinated Polyvinylpyrrolidone (P.V.P.) was supplied by the Radiochemical Centre, Amersham, with an average molecular weight of 40,000.

b. Chromic Chloride ($^{51}CrCl_3$) as supplied by Amersham.

Both of these were injected in the same way as the labelled plasma proteins.

4. Oral Administration of other Isotopically Labelled Compounds

a. P.V.P. with an average molecular weight of 40,000 as above.

b. P.V.P. with an average molecular weight of 160,000, from Amersham.

c. $^{51}CrCl_3$, as above.

These labelled compounds were carefully syringed into the back of the mouth. (See Section VII).

5. Samples

a. Blood

Blood samples were taken from the opposite jugular vein using a 5 or 10 ml vacutainer (Becton, Dickinson & Co., Rutherford, New Jersey, U.S.A.), heparinised for plasma. Four timed blood samples (for plasma) 5 to 15 minutes post injection and additional samples were taken over a 14 day period, at regular intervals. Serum samples were taken at the beginning, the middle and the end of the experiment.

b. Urine and Faeces

The majority of calves were kept in metabolism cages (Section II B) for the collection of urine and faeces.

(i) Collected Together

Using either type of metabolism cage, the total excreted material for each 24 hour period was collected in a bucket, and where necessary the cage and collecting tray and polythene sheet were brushed and washed down. Where calves were contaminated with faeces, this was scraped off and added along with the washings etc., to the collection.

The collection was left to stand to assist in the breakdown of the faecal material.

Twenty four hours later it was homogenised by stirring with a metal rod and rapidly pouring from one bucket to another until all the sediment was broken up and partly suspended. Finally, while the collection was being vigorously stirred, a 500ml wide necked polythene bottle was filled and retained for radioactive determinations.

(ii) Collected Separately

The urine was collected in a bucket, the volume determined using a measuring cylinder and recorded.

The weight of faeces was determined by difference. Small quantities were homogenised ('Ato-mixer', M.S.E., Crawley, Sussex) a known weight of water added if necessary. Larger quantities of fluid faeces were transferred to a bucket and a representative sample obtained as above.

6. Preparation of Samples for Counting

All samples and standards were made up to the final volume (5 or 15 ml, depending on the counter used, and the counting bottles/tubes available) with 0.01N NaOH.

a. Plasma

1ml of plasma was pipetted into a counting bottle and made up to the required volume.

b. Urine

5ml of urine was pipetted into a counting bottle (and made up to 15ml if required).

c. Homogenised Excreted Material (Mixed urine and faeces or just faeces)

It was prepared as follows:

Approximately 5 or 15 ml of homogenate was withdrawn from the polythene bottle or the homogeniser (Section II C 5b (i) and (ii)) containing the representative sample, using an adapted disposable syringe. It was transfered to balanced counting bottles, the final weight to be counted being determined by difference. Initially, four 5ml samples were counted, but subsequently this was changed to one 15ml sample.

d. Radioactive Standards

A 1 in 200 or 1 in 250 dilution of the radioactive sample was prepared, and standards made up for counting as for plasma (see above).

7. Radioactive Measurements (counting of the samples)

These were carried out in one of two well - type sintillation counters.

a. A manually operated counter ('EKCO' Tele-Care, Glasgow) using 5ml counting tubes.

b. An automatic System ('Nuclear Chicago', G.D. Searle & Co., High Wycombe, Bucks) using 5ml tubes or 15ml counting bottles.

Where two isotopic labels were present in the samples, it was necessary to count the samples at two settings, as there was

applying the dilution principle.

(ii) Plasma Disappearance Curve (Q_p)

All subsequent plasma samples were expressed as a percentage of the "t_o" value, and plotted on semi-logarithmic graph paper, for the whole of the experimental period.

(iii) Apparent Plasma Half-Life ($T\frac{1}{2}$) Sterling (1951)

The initial part of the plasma disappearance curve is steep, largely representing distribution, but after the first 2 to 3 days, the decline becomes linear. The slope calculated by linear regression gives the apparent plasma half-life ($T\frac{1}{2}$) expressed in hours or days. It is an approximate index of catabolism.

(iv) Distribution (Sterling, 1951)

The intercept obtained by extrapolating the linear part of the plasma curve to the ordinate is taken as indicating the fraction of the total which would have been present in the plasma at zero time, if equilibrium between the compartments had been instantaneous. The fraction outside the plasma would then represent the extravascular compartment. We can thus derive a ratio of the relative sizes of the two compartments i.e. Extravascular (E.V.) / Intravascular (I.V.).

b. Total Excreted Activity

(i) Retained Activity (Qr)

The excreted activity, on a daily basis was calculated and used to derive the cumulative excreted activity. This was subtracted from the injected dose to give the activity retained in the calf. The retained activity expressed as a percentage of the injected activity, was then plotted, on a semi-logarithmic scale.

(ii) Total Body Half-Life

This was obtained from the slope of the retained activity (Qr) "curve" by linear regression.

(iii) Fractional Catabolic Rate

(Campbell, Cuthbertson, Matthews and McFarlane, 1956)

The daily excreted activity was expressed as a fraction of the total intravascular activity in the middle of the collection period. The mean value for the experimental period was then calculated, leaving out the first 48 hours, when the plasma activity is changing rapidly.

N.B. No correction was made for any change in plasma volume during the experiment.

(iv) Total Body Catabolic Rate

The daily excreted activity was also expressed as a fraction of the retained activity (Qr) in the middle of the collection period. The mean value for the experimental period was then calculated.

always some overlap of the radioactive spectra. Thus with $^{131}I/^{125}I$ it was possible to count ^{131}I alone and then ^{125}I with some ^{131}I overlap. (Similarly with $^{51}Cr/^{125}I$). The degree of overlap in individual samples was subsequently calculated, from the count rate of the ^{131}I or ^{51}Cr standard at its own setting relative to that at the ^{125}I setting and then subtracted.

The part of the radioactive spectrum used for counting i.e. the peak, for the isotopes used was as follows:-

^{131}I	-	0.36 Mev
^{125}I	-	0.035 Mev
^{51}Cr	-	0.32 Mev

Standards and samples were counted twice, the mean count rate being corrected for background. The samples were then corrected for radioactive decay of the isotope, by calculating a decay factor from the change in the standard counts. All counts were expressed, per unit time, and per unit volume or weight.

8. Calculations

a. Plasma Activity

(i) Plasma Volume

The results of the four post-injection plasma samples (expressed as the $\log_{10}$) were used to calculate the "t_o" value by extrapolation. The plasma volume was then calculated by

(v) Extravascular Activity (Qe)

This was obtained by subtracting the plasma activity (Qp) for the middle of the collection period from the retained activity (Qr) to give the extravascular activity Qe.

(vi) Distribution (Campbell et al, 1956)

Where the extravascular activity Qe is maximal, the specific activity of the labelled protein in both compartments is equal i.e. there is no net transfer of labelled molecules in either direction. At this "equilibrium" time (m) the ratio of the extravascular/intravascular activities is equal to the ratio of the sizes of the two compartments i.e. at m ,

$$\frac{Qe}{Qp} = \frac{E.V.}{I.V.}$$

c. Faecal Activity Alone (where determined)

(i) Cumulative Faecal Output

Cumulative faecal output was calculated as a percentage of the injected dose.

(ii) Faecal Clearance

Faecal clearance of plasma (ml/day) was determined by dividing the total faecal activity in each 24 hour period of collection of faeces by the activity per ml of plasma at the beginning of the collection period.

D. HAEMATOLOGY

1. Packed Cell Volume

The packed cell volume (P.C.V.) percentage was determined by the micro-haematocrit method. Heparinised capillary tubes containing the blood samples were sealed at one end, by heat or with Cristaseal (Hawksley & Sons, London) and centrifuged for 6 minutes in a micro-haematocrit centrifuge (Hawksley & Sons, London). The percentage P.C.V. was determined from the scale on a Hawksley Microhaematocrit Reader.

2. Total Protein Determination

The total serum protein concentration was estimated by the biuret method of Welschelbaum (1946).

3. Albumin/Globulin (A/G) Determination

a. The serum protein fractionations were carried out by electrophoresis. Cellulose acetate strips (Oxoid Ltd., London) were carefully soaked in barbitone buffer (pH 8.6), lightly blotted and then laid across the supports of the electrophoresis tank (Shandon Scientific Co., London). The samples (0.003ml of serum) were applied to strips about four centimetres from the cathode end using a micro-pipette. A constant voltage of 150 volts was then applied for one hour, using a Vokam power pack (Shandon Scientific CO., London).

The strips were then removed, dried in a hot air oven

and developed with 0.2% Ponceau S. (G.T. Gurr Ltd., London) in a 3% aqueous trichloracetic acid, for five minutes. The strips were then scanned using a Chromoscan recording densitometer (Joyce Loebl and Co., Gateshead), the results being expressed as a ratio of albumin to globulin.

b. The electrophoretic method (above) was subsequently replaced by the direct spectrophotometric determination of Albumin (Rodkey, 1965).

E. IMMUNOCHEMICAL TECHNIQUES

1. Preparation of Antisera

Antisera were produced in rabbits, New Zealand White, Dutch or Rahere Ginger, weighing 2 to 4 kilograms. For immunisation, a 50/50 mixture of 2% protein solution and complete Freunds Adjuvant (Difco Laboratories, Detroit, Michigan) was prepared and emulsified, manually or using a homogeniser (M.S.E., Crawley, Sussex). The rabbits were then innoculated with 70 - 140 mg of emulsified protein, given intramuscularly, into one or both hind legs. A repeat injection was given 21 - 28 days after the primary inoculation and the rabbit bled 10 days after this. Booster injections were given at 2 months intervals. The rabbits were bled from the marginal ear vein and artery, using Toluene to increase the flow of blood where necessary. By this means, 20 to 40 ml of blood was collected

in universal bottles, allowed to clot overnight at room temperature, and the serum removed. All antisera were stored at -20°C till required.

2. Adsorption of Antisera

For Radial Diffusion (Section II E 5) a pure antiserum is required. To prepare this, rabbits were inoculated with a pure protein fraction (prepared by gel chromatography and / or ion-exchange chromatography, Section III). When the resulting antiserum was tested by immunoelectrophoresis (Section II E 3) antisera raised against immunoglobulins were found to cross react with other immunoglobulins. It was therefore necessary to adsorb the antiserum i.e. to remove the "cross reacting" antibodies, thus rendering the antiserum specific.

Adsorption was carried out as follows:-

a small amount of a suitable protein or protein mixture, (which would precipitate with the cross reacting antibodies, but not with "specific"antibodies) was added to the antiserum and left to stand at room temperature for one hour. The mixture was then stored at 5°C overnight to ensure complete precipitation. Finally it was centrifuged at 5°C for 30 minutes and the supernatant retained. This process was repeated till there was no further precipitation. The specificity of the antiserum was then tested by immunoelectrophoresis and double diffusion

3. Immunoelectrophoresis

a. The Antiserum

For routine examinations the antiserum used was either anti-bovine-serum or anti-bovine-globulin (with some anti-albumin activity). The antisera were stored at -20°C, in 1ml aliquots and thawed as required.

b. Preparation of the Agar

Two litres of barbitone buffer were made up as follows:

20.62gm	sodium barbitone
3.684gm	diethyl barbituric acid
8.2 gm	sodium acetate
200 mg	merthiolate

and the volume made up with deionised water.

To prepare the agar for immunoelectrophoresis, the following mixture was carefully boiled till all the agar had dissolved:

250 ml barbitone buffer (as above)

750 ml deionised water

10 gm agar (Difco Laboratories, Detroit, Michigan)

100 mg merthiolate

The hot agar was poured into universal bottles and when cool, stored at 4°C. Impregnation agar was made by boiling 1 gm of agar in 500 ml of water plus 100 mg of merthiolate and 1 ml of glycine. The mixture was cooled and 500 ml of water added.

It was stored at 4°C, till required. The alcohol cleaned slides were prepared by boiling in the impregnation agar and then drying at room temperature.

c. Electrophoresis

L.K.B. (Croydon, Surrey) equipment was used. The slides were placed in the holders previously fitted into the levelling table. The table was adjusted so that the slides were completely level. 10 ml of the liquid agar was poured onto each set of three slides. After cooling a central trough (6.5 mm by 1 mm) and two wells (1 mm) in diameter and 9 mm apart were cut in each slide. The wells were removed by suction and then filled with antigen using a microlitre syringe. The slide holders were then placed in the electrophoresis tank, the circuit completed with Whatman Chromatography paper (No 3mm) wicks, soaked in the barbitone buffer and the current applied for 1 hour at 250 volts, using a Vokam power pack (Shandon Scientific Co., London).

d. Diffusion

When electrophoresis was complete, the central troughs were removed with an agar knife and filled with antiserum. The slides were then incubated overnight at 37°C. In some cases, it was necessary to allow the slides to diffuse for up to 48 hours. The slides were examined at this stage and the results

recorded. In some cases they were also photographed.

e. Staining

The slides were washed in saline for one hour and then overnight (for 15 hours). They were finally washed in water for at least an hour. The washed slides were then dried in an incubator at 37°C. The dried slides were stained with saturated AmidoBlack for 5 minutes and then washed in an Acetic Acid/Methanol washtill the unbound dye was removed. The slides were finally dried and stored.

4. Immuno Double diffusion

a. The Antiserum

The antisera used were the same as those used in Section II E 3 (see above).

b. Preparation of the Agar

15 ml of liquid agar (as in Section II E 2) was poured into a Petri dish and any surface bubbles removed. The desired pattern of wells was then cut using one of two cutters (Shandon Scientific Co., London) to provide a central well for the antiserum and six peripheral wells for the samples.

c. Diffusion

The wells were carefully filled using fine Pasteur pipettes, and the plate incubated for 24 to 72 hours, at 37°C, to allow the precipitin lines to develope.

d. Staining

The plates were washed in saline for 48 hours, with three changes, and in water for a further 48 hours. The gel was then either removed from the Petri dish and dried on a glass slide, or dried in situ. The dried gel was then stained with saturated Amido Black for 15 minutes, unbound dye then being removed in an Acetic Acid/Methanol wash. The slide or plate was finally dried and photographed or stored.

5. Single Immuno Radial diffusion

The method used was basically that of Mancini, Carbonara and Heremans (1965).

a. The Antiserum

Before the estimations could be carried out, it was necessary to determine a suitable dilution of the specific antiserum and of the samples, where necessary. To this end, trial runs were carried out, using a divided Petri dish. Four samples likely to represent the range of concentrations to be encountered in the samples to be subsequently tested were allowed to diffuse in agar containing the undiluted antiserum and three dilutions of it. If at an apparently optimum antiserum concentration, as judged by the size and clarity of the precipitin ring, one or more samples were too concentrated, i.e. gave large rings, with a risk of overlap, these were also suitably diluted.

b. Preparation of the Agar

The agar was prepared as in Section II E 3b, except that the concentration of agar was increased from 1% to 3% i.e. 30 gm of agar were used. It was stored in 15 ml aliquots at 4°C, till required. Prior to pouring the plates, a bottle of agar was melted and then placed in a water bath at 60°C. The antiserum was diluted (as above) up to a final volume of 7 ml and placed in a water bath at 55°C.

A glass plate was prepared, by boiling it in impregnation agar (Section II E 3b) and allowing it to dry. A second plate was coated with silicone jelly and the Perspex mould placed on top. The top and inside surfaces of the mould were also coated with the jelly. Finally the impregnated plate was placed on top and the two plates with the mould between, clamped together.

When the temperatures in the water baths were constant, 7 ml of the agar was added, with a warmed pipette, to the antiserum. The mixture, after gentle shaking was then pipetted carefully into the mould. After 15 minutes, when the gel had set, the mould and the siliconised plate were carefully removed. 12 or 16 wells (2 mm in diameter) were then cut in the plate and the agar plugs removed by suction.

c. Diffusion

0.4µl of each sample was applied with a microlitre syringe

(Griffin & George, East Kilbride) along with a standard and three or more dilutions of the standard. When the samples had diffused into the gel, one of two possible procedures was carried out:

(i) The plate was placed in a shallow tray and covered with 2 cm of liquid paraffin in which a few crystals of thymol had been dissolved. The plate was then incubated at 37°C till diffusion was complete. Diffusion was judged to be complete when the largest precipitin rings did not change in diameter between two readings, 24 hours apart. The diameter of the rings was measured using a magnified scale (Telemaster, Verebes & Co., London), two readings being made at right angles, and the mean value taken as the diameter.

(ii) the plate was placed in a moist airtight container and left at room temperature till diffusion was complete.

d. Staining

The washing procedure was carried out as for double diffusion (II E 4d). To prevent the agar from cracking when the slides were drying, a wet filter paper was placed on top of the slides and a hole pierced with a needle over each well (Yamamoto, Hashimoto and Yokoyama, 1968). The plate was dried at 37°C and the dry filter paper carefully removed. The plate was then stained, washed and dried. In some cases, the diameter of the rings was again measured.

e. Calculation of Results

All the results were expressed as the square of the ring diameter (d^2). For the standards, d^2 was plotted against concentration (mg/ml), determined by the buiret method (Weischelbaun, 1946). The concentrations of the other samples were then read from this graph. Any sample above the range covered by the standards was diluted and then the quantitation repeated.

F. EVANS BLUE (T.1824) PLASMA VOLUME

The proceedure followed was similar to that used for the labelled protein. Two syringes were filled and weighed, 2 ml (of a 1% solution in saline) for the calf and 0.5 ml for the standard. A 10 ml pre injection heparinised blood sample was taken. The calf was then injected via the jugular catheter. Four 5 ml heparinised blood samples were taken from the opposite jugular vein, 5 to 15 minutes post injection, using vacutainers. The samples were centrifuged at 3,000 r.p.m. (M.S.E., Crawley, Sussex) and then diluted to 2 or 4 ml with saline, for reading. The standard was made up to 500 ml with saline and 1 ml of it added to 1 ml of the pre injection plasma. This was diluted to 4 ml with saline, if required.

During the studies, two methods were used to read the samples:

the prepared 2 ml samples and standard were read in an

E.E.L. Colorimeter (Baird and Tatlock, Glasgow) using a 607 filter.

Subsequently, the colorimeter method was replaced by the use of a spectrophotometer, the SP 600 (Pye Unicam, Cambridge). The prepared samples, this time diluted to 4 ml were read at a wavelength of 620mμ.

The plasma volume was calculated by applying the dilution principle, after the concentration of Evans Blue at "t_o" had been determined by extrapolation (the plasma concentration being expressed as the $\log_{10}$).

G. STATISTICAL METHODS

The statistical methods used were those described by Snedecor (1956) and Bishop (1966). The exponential phase of the radioactivity curves was calculated by linear regression analysis. Correlation coefficients of $r = >0.95$ were accepted. Deviations from the mean were expressed as the Standard Deviation (S.D.) and the Standard Error (S.E.) of the mean. The "t" values were taken to be significant at $p < 0.05$.

SECTION III

THE PREPARATION OF BOVINE FAST IgG (IgG_1) IMMUNOGLOBULIN

INTRODUCTION

The investigation of the distribution of Bovine Fast IgG in the neonatal calf required the preparation of a pure protein fraction, undenatured, in quantities suitable for labelling with the isotopes of iodine, i.e. up to 500 mg. Pierce (1966), Schultze and Heremans (1966) and Weir (1967) give the following methods for the separation and purification of plasma proteins.

Salting out, which may be combined with other methods

Electrophoresis - in part superceded by ion-exchange chromatography

Ultracentrifugation - differentiating between proteins with different sedimentation values.

Isolation of Specific Antibody - from complexes of antigen and antibody

Molecular Sieve Chromatography

Ion-exchange Chromatography

Different combinations of two or more methods may also be used.

These methods were then considered in the light of the three requirements already stated above. Salting out was excluded because of the risk of denaturation. Electrophoresis and ultracentrifugation are limited by the amount of sample that

can be separated and present problems with regard to obtaining a sufficiently pure prepatation (as does salting out). The isolation of specific antibody carries the risk of alteration of the molecules and a specific antibody was not required. The object was to obtain an IgG fraction containing a whole range of specific antibodies. The objections raised to these methods of separation are not exhaustive but they are sufficient to justify concentrating on the two remaining methods - that of molecular sieve chromatography and ion-exchange chromatography.

Molecular sieve effects in gels were observed by Synge and Tiselius (1950) in agar but Lathe and Ruthven (1956), using starch, were the first to demonstrate that the technique of molecular sieve chromatography can be applied to biological compounds. The breekthrough in this field came with the development of a new type of gel prepared by cross-linking dextran to form a macromolecular network of great stability (Porath and Flodin, 1959). They coined the name ' gel filtration ' to distinguish it from other forms of chromatography. Many other workers however prefer the term molecular sieve chromatography (Anderson and Stoddart, 1966).

The gel introduced by Porath et al (1959) had a high degree of cross linkage and thus lent itself to the desalting of proteins (Porath, 1960, Flodin 1961, Granath and Flodin, 1961, Porath 1962 and Andrews 1966). Porath et al (1959) considered

that molecules were separated according to their size, but a variety of other factors were subsequently shown to influence separations. Aromatic and heterocyclic compounds were found to be adsorbed (Gelotte, 1960) but other effects could be prevented by the use of suitable buffers. These factors were further investigated by Glazer and Wellner (1962), Marsden (1965), Andrews (1966) and Stevensen (1968).

As a result of the pioneer work of Porath and Flodin, a whole range of dextran gels with varying degrees of cross-linkage were made commercially available under the trade name of Sephadex. They are designated G-10, G-25, G-50, G-75, G-100, G-150 and G-200 in order of decreasing cross-linkage and increasing water regain and fractionation range.

Porath (1962) reported the ability of Sephadex G-200 to divide serum into three main fractions (peaks) on a molecular weight basis. Similar results were obtained by Flodin and Killander (1962), Gelotte, Flodin and Killander (1962), Killander and Flodin (1962), Fahey and McLaughlin (1963), Killander and Hogman (1963), when separating 7S and 19S human blood group antibodies, Killander (1963), Hogman, Killander and Johansson (1964), Killander (1964), Rothstein (1965) and Roskes and Thompson (1963) who used the G-200 separation to differentiate between normal and pathological sera.

The experimental details vary from worker to worker but are usually based on the pioneer work of Porath and Flodin (1959), Porath (1960), Flodin (1961), Flodin and Killander (1962) and Porath and Flodin (1962). In later reviews, Andrews (1964), Selby and Maitland (1965), Andrews (1966) and Bailey (1967), the technique was further developed.

Since Lathe and Ruthven (1956) used starch columns to determine the molecular weights of solutes, other workers have used agar (Andrews, 1962), polyacrylamide gel (Hjerten, 1962) and Sephadex (Whitaker, 1963, Ackers 1964, Andrews 1964 and Andrews 1965 and 1966) for this purpose. This particular application, developed on an empirical basis, has been hampered by disagreement and lack of understanding with regard to the fundamental mechanisms which bring about the molecular sieve effect.

Two main theories have evolved, both having their adherents. Lathe and Ruthven (1956) first suggested that the extent of penetration of the stationary phase depends primarily on the molecular weight of the solutes concerned. This idea was developed further by Porath and Flodin (1962) who concluded that Sephadex acted as an almost perfect molecular sieve, the optimum range of molecular separation for a particular gel being determined by the extent to which it swells in the solvent.

This is determined by the amount of cross-linkage. Porath (1963) elaborated these ideas. The other theory was put forward by Polson (1961) who considered, from his work with granular agar, that the degree of separation is largely dependent on differences in diffusion coefficients. Experimental evidence has been put forward in support of both concepts. Exclusion from the matrix was favoured by Squire (1964), Laurent and Killander (1964) and Andrews (1966). Steere and Ackers (1962) supported the concept of restricted diffusion in agar gel columns and subsequently Ackers (1964) obtained results suggesting that both theories should be considered, depending on the degree of cross linkage of the Sephadex. In reviews of the subject Anderson and Stoddart (1966) and Ogston (1966) concluded that practical application had inevitably outstripped theoretical treatment and that it was a subject of great difficulty.

Apart from Sephadex, other media have been used . e.g. polyacrylamide gel and polyvinyl alcohol (Laurent and Killander, 1964), glass (Pederson 1962 and Haller 1965) and agar and starch (as above).

The application of ion-exchange chromatography to the separation of proteins was limited by the need for a very large surface area and the instability of the protein molecule (Peterson and Sober, 1959 and 1960a, Huisman 1960, Peeters 1960

and Tiselius 1967). Porter (1954), referring to the use of Amberlite IRC-50 resin in particular, concluded that the method was confined to proteins most resistant to denaturation. Bowman (1955) and Bowman and Westland (1956) used Dowex 2. An alternative technique using Calcium Phosphate as adsorbent was reported by Swingle and Tiselius (1951) and developed by Tiselius , Hjerten and Levin (1956) and Hjerten (1959).

Ion-exchange was made generally applicable to the separation of proteins by the introduction of substituted celluloses, anionic (DEAE) and cationic (CM) exchangers (Sober and Peterson, 1954) thus reducing the risk of changes in the configuration of the protein molecule. (Peterson and Sober, 1956). Their application to plasma proteins was described by Sober, Gutter, Wycoff and Peterson (1956) and Sober, Wycoff and Peterson (1957). The initial observations were included in a comprehensive review of ion-exchange chromatography by Moore and Stein (1956). Subsequently Sober and Peterson (1957) and (1958) and Fahey, McCoy and Goulian (1958) developed the technique further. The initial work was reviewed and consolidated by Peterson and Sober (1960a).

The theoretical aspects were also considered by Peterson and Sober (Sober and Peterson, 1958; Peterson and Sober, 1960a and Peterson and Sober, 1962), as well as by Peeters (1960).

The forces causing the binding of the plasma proteins to the cellulose particles bearing the opposite charge are electrostatic, although other forces are also believed to play a part. The bonds can be broken or disconnected by a small change in the pH or an increase in the ionic strength. The former reduces the number of charges on the protein molecule while the latter will decrease the effectiveness of the existing bonds by promoting their dissociation. Thus Sober and Peterson (1958) found that, as a rule, proteins with high iso-electric points were eluted earlier. A number of factors, pH dependent differences, the presence of complexes, specific ion-electrostatic affinity for the adsorbent and the size of the protein molecule, may tend to alter this situation.

The two types of gradient described by Alm, Williams and Tiselius (1952) were both applied by Sober and Peterson (1958). Gradient elution provided the required range of eluting power without sharp changes in the composition of the eluant. Stepwise elution on the other hand gave a poorer resolution and made interpretation hazardous, with the appearance of extra peaks. It requires preliminary separations to determine the best conditions.

Gradient elution was improved by the introduction of the varigrad (Peterson and Sober 1959 and 1960b) which enabled

small changes to be made in specific portions of the gradient. The equipment was improved by Peterson and Rowland (1961) and used by Peterson and Chiazze (1962) to standardize the technique.

Many workers have used DEAE cellulose for the separation of serum, plasma and plasma protein fractions. Humphrey and Porter (1957) obtained 4 γ globulin components. Sober and Peterson (1958) recovered 90% of the total γ globulin in the breakthrough peak. These findings were confirmed by other workers (Fahey, McCoy and Goulian, 1958, Fahey and Horbett 1959, Prager, Speer, Hill, Williams and Goerner, 1959 and Fahey 1960). Sober et al (1958) and Peeters (1960) considered that the method was most suitable for large scale separation under mild conditions. Strauss, Kemp, Vannier and Goodman (1964) increased their yield by carrying out initial precipitation with Sodium Sulphate. Other workers scaled it down for diagnostic purposes. (Fahey et al 1958 and Prager et al 1959). Cooke, Tombs, Weston, Souter and Maclagan (1957) obtained complex peaks when separating serum.

Pure γ globulin was obtained by Ziff and Lospalluto (1959), Levy and Sober (1960) and by Stanworth (1960), who used a batch proceedure. Work by Fahey et al (1958), (1959) and (1960) and Fahey (1960) on the separation of 7S and 19S

antibodies was confirmed by Goodman, de St Cyr, Cleve and Grabar (1960), Goodman, Fahey and Malmgren (1960) and Albert and Johnston (1961).

Carroll, 1961 applied the technique to the separation of colostral proteins using stepwise elution, following the method of Yaguchi, Tarassuk and Hunzik (1961) for separating milk proteins.

Gelotte, Flodin and Killander referred to the introduction of DEAE Sephadex A-50 which became commercially available in 1961. It has about 3 times the capacity of DEAE cellulose. With the recognition of the complex nature of the immunoglobulins, Vaerman, Heremans and Vaerman (1963), found that it was only possible to obtain 50% of the serum IgG in a pure form, in the breakthrough peak. Goodman (1964) while reviewing the method, showed how initial Ammonium Sulphate precipitation could increase the yield substantially.

The two methods of separation, molecular sieve chromatography and ion exchange chromatography have both been applied to the separation of bovine serum and colostral whey proteins by a number of workers.

Carroll (1961) used DEAE cellulose and stepwise elution as performed by Yaguchi et al (1961) to examine colostral whey and whey prepared from "drying off" secretions and mastitis milk.

The first four peaks all contained immune globulins.

Murphy et al (1964a) used two different gradient systems to compare the Gamma globulin components of bovine serum with those of bovine colostrum and dry secretion. Using DEAE Sephadex A-50, their first peak consisted of IgG_2 and the second, eluted as the gradient predominated was mostly IgG_1.

The same workers (Murphy, et al, 1964b) to prepare bovine IgM, used a Sephadex G-200 column (7 x 70 cm) separating bovine serum into three major groups on a molecular weight basis, as in man. Subsequently they (Murphy et al, 1965) applied both techniques in an investigation of the physical heterogeneity of Bovine γ - Globulins. They showed that their ion-exchange chromatograms of bovine serum were analogous to the chromatograms obtained with human serum, consisting of 3 main peaks. The first (breakthrough) peak consists of unbound protein in the void volume (IgG_2). The second peak, larger than in man, contains a faster migrating IgG (IgG_1) and transferrin. The third peak contains albumin and other plasma proteins.

MATERIALS AND METHODS

A. Starting Materials

1. Neonatal Calf Serum

Blood was obtained from a three day old Ayrshire bull calf, supplied by Dr. I. Selman, Department of Veterinary Medicine, University of Glasgow. The calf had received colostrum and at 48 hours post partum its serum gave a Zinc Sulphate reading (McEwan, 1968) of 36 units. The blood, in 100 ml beakers, was allowed to clot at room temperature. The clots were 'ringed' and the serum removed the following day. It was centrifuged at 3000r.p.m. and the supernatant split up into 20 ml aliquots in universal bottles. The total protein concentration and A/G ratio were determined, and the aliquots stored in the deep freeze at -20°C until required.

Results of protein concentration determinations:

T.P.	-	7.4 gm/100ml
A/G ratio	-	0.38

2. Colostral Whey

This was prepared from fresh bovine colostrum supplied by Dr.I.Selman. 50 ml batches of colostrum in large centrifuge tubes were raised to 37°C, in a water bath and 0.7 ml of commercial rennet added (J.Jaap & Co., Glasgow). When the

casein had set and the whey was beginning to separate, it was centrifuged at 3000r.p.m. enabling the whey to be separated from the clot and from the layer of fat which formed on top of the whey. The whey thus obtained was stored in 20 ml aliquots at -20°C. It had a Total Protein of 9.6 gm/100ml and an A/G ratio of 0.21.

B. Molecular Sieve Chromatography

The medium used was Sephadex G-200, supplied by Pharmacia (G.B.) Ltd., 75 Uxbridge Road, London W.5. The starting material i.e. the sample, was neonatal calf serum (as above). A 0.1M Tris - HCl - 0.5M NaCl buffer, pH 8.0 was used for swelling the Sephadex and as eluant. Sodium Azide (0.02%) was added to prevent bacterial growth (Williams and Chase, 1968). 25 gm of Sephadex (an excess, calculated from the water regain value of 30 to 40 ml/gm) was slowly added to 1 litre of buffer with continuous stirring. After 24 hours at room temperature, the buffer on top of the partly swollen gel was removed, along with any fine particles. More buffer was added and mixed with the gel. The process was repeated at 48 and 72 hours. After the final mixing, the gel was ready for packing the column.

All column work was carried out at a temperature of 8 - 10 °C. This temperature was maintained by the use of a water jacket and / or a 'cold' room.

1. Column Preparation

The glass column, 100 cm by 3 cm diameter was fitted with two rubber bungs, pierced by portexconnectors (Portland Plastics Ltd., Hythe, Kent). At the bottom of the column a pad of glass wool had beentrapped on top of the bung, thus providing a mesh which would retain the Sephadex but allow free passage of buffer into tubing attached to the connector (with little or no dead space, i.e. space which would allow mixing of the protein fractions, as they came off the column).

A little pre-swollen Sephadex was then poured gently into the column, care being taken to avoid air bubbles. If there was no evidence of leakage of the Sephadex, the column was completely filled and the level of the out-let tubing adjusted so that the head of pressure on the column did not exceed 10 cm. Over a period of 6 to 7 hours the column was periodically topped up after removal of the layer of buffer which formed at the top of the column, due to gel packing down. When the level of the gel in the column had ceased to fall, the column was connected to a 1 litre reservoir containing more of the eluant buffer, at constant pressure. The level of the reservoir and the outlet were again adjusted and the column left to flow under gravity overnight. If the level of the bed had fallen overnight, more Sephadex was added to bring the

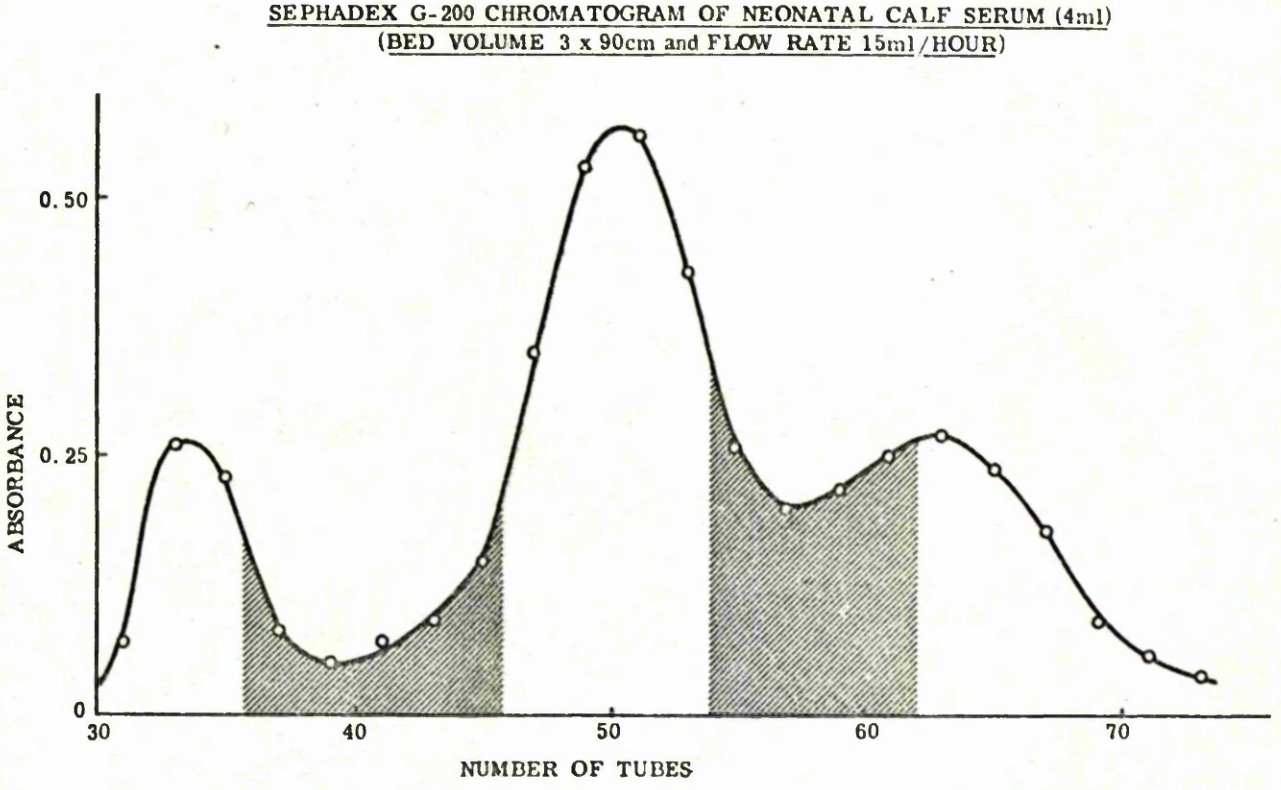
height of the Sephadex bed to about 90 cm. Finally, before applying the sample, the top of the bed was stabilised by the application of a piece of filter paper, cut to fit the column.

2. Column Operation

A 4ml sample of the calf serum was carefully layered on to the top of the column, after removal of the buffer present there. When the sample had passed into the gel, a layer of buffer was similarly layered on top. In both cases, great care was taken to ensure that the top of the bed was not disturbed. If this did occur, a small glass rod was used to thoroughly mix the sample with the Sephadex at the top of the column and when it settled down again buffer was layered on top, as before.

The column was reconnected to the reservoir and the outlet connected to the Fraction Collector (L.K.B., Croydon, Surrey). The effluent from the column was then collected in 4ml fractions over the next 48 - 72 hours. The total duration of the separation depended on the flow rate which was found to vary from column to column. While theoretically, once a column is set up, it can be used time after time, in practice it was found that after being used 2 or 3 times, for no apparent reason the flow rate gradually became so slow, that the column had to be emptied and re-packed. Occasionally columns accidently ran dry, also necessitating repacking.

Figure 2


SEPHADEX G-200 CHROMATOGRAM OF NEONATAL CALF SERUM (4ml)
(BED VOLUME 3 x 90cm and FLOW RATE 15ml/HOUR)
ABSORBANCE
0.50
0.25
0
30
40
50
60
70
NUMBER OF TUBES

The 4 ml fractions were removed in batches and their protein content examined at 280mμ in an S.P. 500 spectrophotometer (Pye Unicam, Cambridge). They were stored at 5°C until the separation was complete. A graph of absorbance against effluent volume was plotted. The separation was continued till no more protein could be detected. The chromatogram (Fig 2) obtained showed the characteristic 3 peaks (see Introduction). The fractions containing the top of the middle peak were then selected by estimating the amount of overlap, seen in Fig 2 as the shaded area, either side of the middle peak. These fractions were then excluded and the remaining fractions bulked.

3. Concentration of Fractions

The fractions were concentrated by making use of the osmotic properties of Sephadex G-25 (Flodin, Gelotte and Porath, 1960, and Deutsh, Levere and Levine, 1963). The Sephadex (1 gm per 5 ml to be concentrated) was placed in a Buchner funnel, on top of a moist filter paper. The bulked fractions from the column were poured onto the Sephadex while stirring, until all the dilute protein has been added. The protein was then allowed to drip through into a flask with a side arm. After five minutes, or when the Sephadex has become semi-solid, negative pressure (using a filter pump) was applied to remove the rest of the concentrated protein. To minimise the production of bubbles the pressure was carefully controlled. The process

was repeated until the volume had been reduced to about 10 ml. The concentrated fraction was then examined by Immunoelectrophoresis and Double Diffusion, prior to storage at -20°C (see Section II E 3 and 4).

4. Repeat Separations

For subsequent separations the position of the middle peak was ascertained from the position of the troughs on either side of it, and the equivalent fractions kept for concentration. A further forty one G-200 separations were carried out in the manner indicated.

The results of the Immunoelectrophoretic examination were as follows:

19 samples gave one line corresponding to Fast IgG

15 samples gave no line (i.e. negative)

6 samples showed Fast IgG contaminated with other plasma proteins

2 samples showed contaminating proteins and no Fast IgG

The 15 negative samples were then re-examined by Double Diffusion. Ten of them gave one line i.e. a single protein fraction present, most likely to be Fast IgG. The other five showed, in addition to this line, evidence of contaminating proteins. The samples that were negative on Immunoelectro-

Table I

Examination of the grouped IgG_1 Fractions (after concentration) by Immunoelectrophoresis and Double Diffusion

Group	Immunoelectrophoresis	Double Diffusion
A	Pure	Trace impurity
B	Pure	Trace impurity
C	Slight impurity	Trace impurity
D	Slight impurity	Trace impurity
E	Pure	Trace impurity
F	Pure	Trace impurity
G	Slight impurity	Contaminated
H	Pure	Not tested
I	Slight impurity	Not tested

phoresis were probably too dilute.

All the samples were then grouped together (see Table 1) and concentrated to a volume of about 10 ml. The resultant concentrates were again examined by Immunoelectrophoresis. The results were disappointing and rather variable. Of the bulked "pure" fractions Group C now showed definite signs of other protein fractions being present and Groups D and G showed a slight impurity to be present. One of the impure groups was now showing up as a pure fraction. When Double Diffusion was carried out on the same samples there was the suggestion of a trace impurity in all of them, except G, which was very definitely contaminated. The concentration of the main contaminating protein appeared to be poised at a level where its presence might or might not be detected by Immunoelectrophoresis, whereas it was always detected by Double Diffusion. Thus, although results from individual separations had suggested that it might be possible to obtain a pure fraction using G-200 alone, this was clearly seen to be impossible.

The presence in the middle G-200 peak, along with the Fast IgG, of the contaminating protein indicated that it had a similar molecular weight (assuming similarly shaped molecules, and no "adsorption" of either molecule by Sephadex).

Provided there was some difference in molecular weight, it might be possible to gradually separate the two proteins by rechromatography, until a sufficiently pure IgG preparation was obtained. Apart from the time and labour involved in such a procedure, it might also suffer from an increased risk of denaturation and a loss of protein each time it was concentrated. It was therefore decided to try and separate the proteins on an ionic basis, using ion-exchange chromatography.

C. Ion-Exchange Chromatography

The medium used was Sephadex DEAE A-50 supplied by Pharmacia (G.B.) Ltd., 75 Uxbridge Road, London W.5. The bulked IgG fractions, obtained by molecular sieve chromatography were grouped:

A, B, E, F and H in Group 1
D and G in Group 2
C and I in Group 3

The three groups were then concentrated down to about 20 ml with Sephadex G-25, to be used as starting material for the ion-exchange separation.

A 0.01M Phosphate buffer, pH 8.0 was used to swell the DEAE Sephadex and as the initial eluant. Subsequently an approximately linear gradient was applied, using equal volumes

of the 0.01M buffer and a 0.5M Phosphate buffer, pH 8.0 i.e. a molarity gradient, at constant pH. The litre reservoirs, containing 500 ml of the respective buffers were connected by a siphon so that a drop in the level of the low molarity buffers, connected directly to the column, caused the difference in volume to be made up by the high molarity buffer. Six gms of the Sephadex was slowly added to 500 ml of the starting buffer (0.01M) with continuous stirring. The buffer was then changed 3 times in 24 hours, to ensure complete equilibration. The method employed for swelling the gel was basically the same as that used for the G-200 Sephadex. After the final mixing the gel was ready for packing the column.

1. Column Preparation

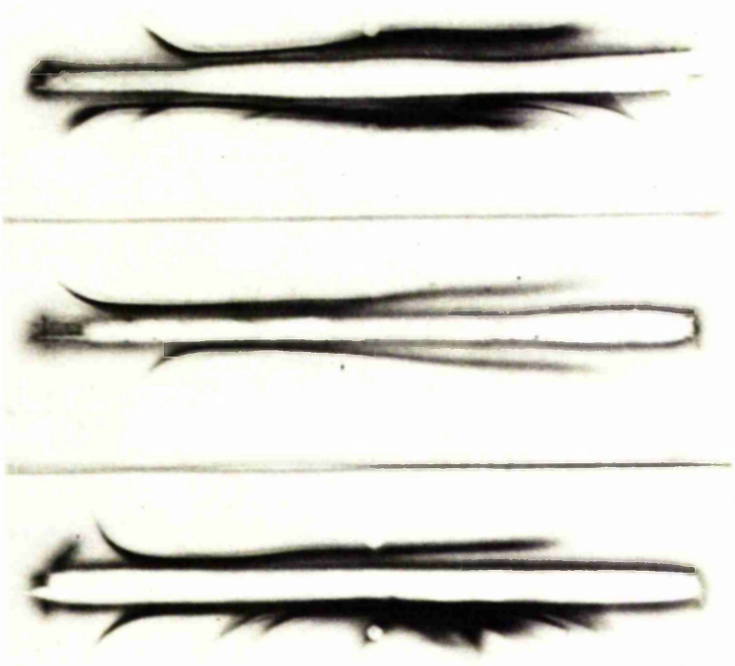
The glass column, 40 cm by 3 cm diameter was assembled, as for G-200. The column was packed in a similar way, allowing a head of pressure up to 30 cms. It was complete in 2 to 3 hours, to give a final volume of 30 cm by 3 cm diameter. For other details, see Section II B 1, above.

2. Column Operation

The sample was applied and after the buffer had been layered on top, the column was connected to the 0.01M phosphate reservoir, and to the fraction collector. The

Figure 3

Immunoelectrophoretic Comparison of Bovine Fast and Slow IgG Fractions



B.S. - Bovine Serum

A.B.G. - Anti-bovine-globulin (with some anti-albumin activity)

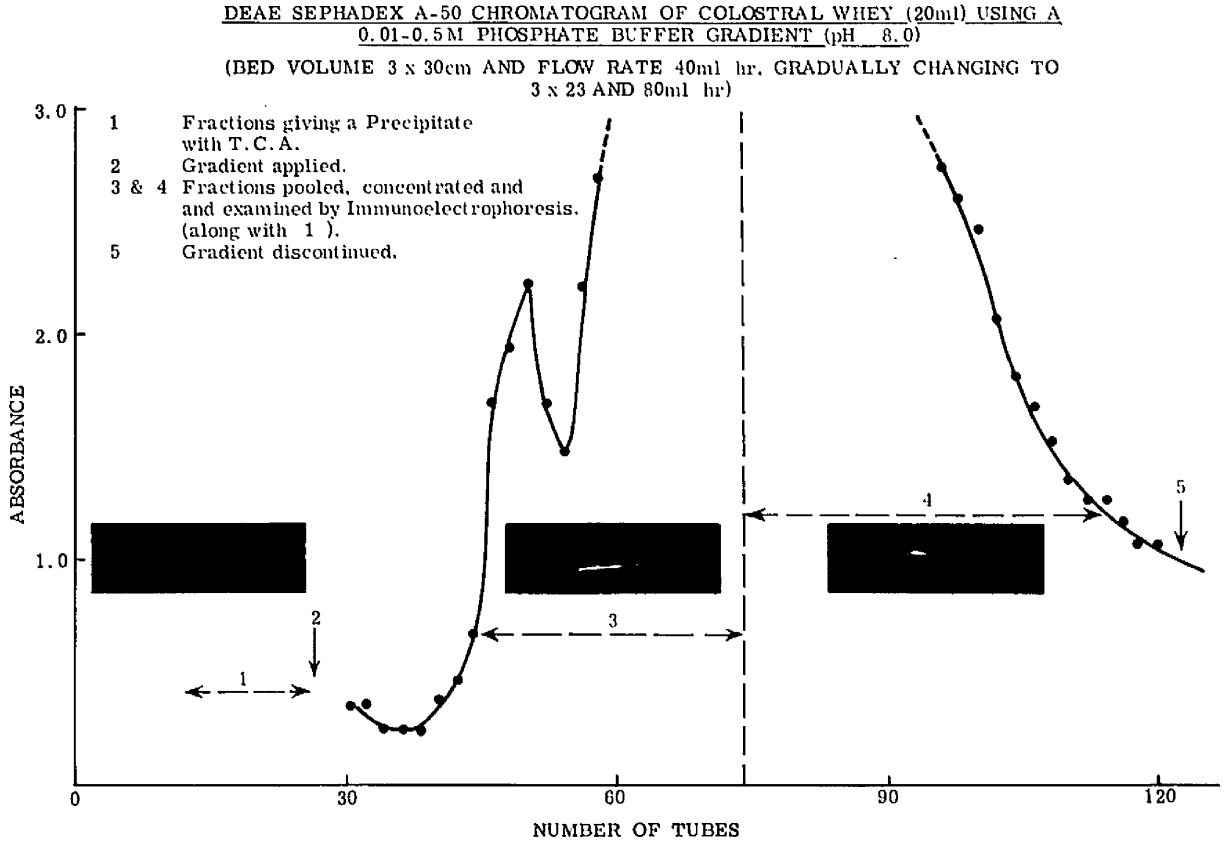
buffer in this reservoir was stirred continuously. The 5ml fractions were tested for protein, with 5% T.C.A., as they were collected and a slight break through peak detected. When this peak had been eluted, the molarity gradient was applied, to elute the bound protein. Prior to this, considerable shrinkage of the bed volume occurred, due to the increase in molarity resulting initially in a reduced flow rate. When the bed volume stabilised the flow rate increased, and the second peak was eluted, its presence being detected with 5% T.C.A. All the fractions were then read in the SP 500 at 280 mμ and a chromatogram plotted. The third peak was not collected, the separation having been stopped while the remaining protein was still bound to the Sephadex.

3. Concentration of Fractions

The small break through peak was concentrated with G-25. The second, much larger peak was divided into two parts and each part concentrated. The concentrated fractions, 5 - 10 ml. were then checked by Double Diffusion and Immuno-electrophoresis. Both peaks were found to be IgG fractions, the first designated Slow IgG (IgG_2) and the second Fast IgG (IgG_1), see Figure 3. Groups 1, 2 and 3 were all separated and yielded the following concentrations of Fast IgG.

Group 1 ..... 20 ml of 2.5%

Figure 4


DEAE SEPHADEX A-50 CHROMATOGRAM OF COLOSTRAL WHEY (20ml) USING A 0.01-0.5M PHOSPHATE BUFFER GRADIENT (pH 8.0)
(BED VOLUME 3 x 30cm AND FLOW RATE 40ml hr. GRADUALLY CHANGING TO 3 x 23 AND 80ml hr)
1 Fractions giving a Precipitate with T.C.A.
2 Gradient applied.
3 & 4 Fractions pooled, concentrated and and examined by Immunoelectrophoresis. (along with 1).
5 Gradient discontinued.
ABSORBANCE
3.0
2.0
1.0
0
30
60
90
120
NUMBER OF TUBES
1
2
3
4
5

Group 2 ..... 10 ml of 1.6%

Group 3 ..... 10 ml of 1.8%

4. Repeat Separations

Although this method yielded Fast IgG suitable for labelling with the isotopes of iodine, it suffered from the major disadvantage that the whole process took up to three months to complete. The limiting factor was the G-200 separation, taking 2 to 3 days for the separation of only 4 ml of serum. To overcome this difficulty, two changes were made. The order of chromatographic processes was reversed i.e. the DEAE separation was carried out first and the starting material was changed from neonatal calf serum to colostral whey.

Four DEAE separations were carried out as described, using 20 ml of colostral whey instead of the partly purified IgG fraction (Fig 4). The large second peaks were collected, concentrated separately and examined by immunoelectrophoresis. The 10 ml concentrated fractions were then separated on Sephadex G-200 as before. This time, the first and largest peak was concentrated and examined by Immunoelectrophoresis. It was found to contain Fast IgG, a very slight trace of another fraction sometimes being detected. By carrying out two separations at any one time, the length of time required for the preparation was reduced to three weeks.

DISCUSSION

The final method of preparation of Fast IgG described in II C 4 above, proved to be perfectly satisfactory. It could however conveniently be scaled up to avoid the duplicate DEAE A-50 separations. Thus, depending on the concentration of Fast IgG in the starting material, the desired yield , and, also assuming that large enough columns are available, the whole process should be reduced to a single large scale separation on DEAE Sephadex A-50, followed, after the required fractions have been concentrated by ultrafiltration (Millipore U.K., London) by a single separation on Sephadex G-200. Nansen (1970) assumed that it would be difficult to avoid a slight admixture of the two IgG subclasses when separating serum containing IgG_2. He therefore prepared IgG_1, from serum which did not contain IgG_2, obtained from a cow with lymphatic leucaemia and chronic purulent broncho pneumonia (i.e. the breakthrough peak was absent when the serum was chromatographed on DEAE Sephadex A-50). However, using the method suggested above, it should be possible to obtain a sufficiently pure Fast IgG (IgG_1) preparation, using adult bovine serum as the starting material, instead of the calf serum and colostral whey used in these studies.

SUMMARY

The application of molecular sieve chromatography to the preparation of Bovine Fast IgG (IgG_1), failed to yield a sufficiently pure fraction. When the IgG_1 enriched fractions thus obtained were subjected to ion-exchange chromatography, the majority of the contaminants were removed. The method of preparation was improved by the use of colostral whey as the starting material and by carrying out the ion-exchange separation first. A method had thus been found that will provide Fast IgG, suitable for labelling with the isotopes of iodine.

SECTION IV

THE METABOLISM OF BOVINE FAST IgG (IgG_1)

INTRODUCTION

The existence of an extravascular "circulation" of plasma proteins has been known for a long time. Lewis (1927) showed that injured capillaries became highly permeable to proteins. Krogh (1930), while recognising the permeability of liver and intestinal capillaries, considered the other capillary beds to be impermeable to colloids. Field and Drinker (1931) and Drinker and Field (1933) suggested, on the basis of lymphatic studies, that capillaries universally leak protein. Landis (1934) considered that the presence of blood protein in the lymph makes it unlikely that the capillary endothelium is totally impermeable to plasma proteins. He suggested that his finding that 95% of the plasma proteins are retained, could be most readily explained in terms of the presence of a few large pores, permitting the leak of albumin and globulins, in addition to regional variation. Krogh (1930) found that colloids failed to return directly to the circulation, but instead returned via the lymphatics. Subsequent work concentrated on an investigation of these "pores", using dextrans of known molecular weight and isotopically labelled plasma proteins. The results of Wasserman and Mayerson (1951) using ^{131}I albumin leave no doubt that in healthy animals, the capillaries are partially permeable to albumin, some more than others. Bollman (1953) and Pappenheimer (1953) were of a similar

opinion. Manery (1954) while accepting that permeability had been amply demonstrated, suggested that workers varied in their views with regard to the quantitative significance of this "leak".

Grotte (1956), Grotte, Juhlin and Sandberg (1960) and Mayerson, Wolfram, Shirley and Wasserman (1960) produced evidence for two sets of discrete pores, operating completely independently. However, they disagreed as to the upper limit of the small set. Mayerson et al (1960) found evidence of the passage of molecules up to a mol. wt. of 250,000 in contrast to pores of 35 to 45 ^{o}A (Grotte, 1956) which allow passage of molecules with a 30,000 to 40,000 molecular weight. Subsequently Renkin (1954) and Renkin and Garlick (1969) found 35 to 40 ^{o}A pores (i.e. a steep fall for molecules with a molecular weight of between 10,000 and 40,000). Above 100,000 (70^{o}A) Renkin et al, 1969, found little decline which could be accounted for on the basis of increasing molecular weight. In the discussion of their paper, a mol. wt. of 80,000 was given as the upper limit of the small pores. There is thus some doubt about the size of the small pores.

With regard to the large pore system it is generally accepted that molecules with a molecular weight of 412,000 to 500,000 or larger will pass through (Mayerson et al, 1960, Grotte 1960 and Renkin and Garlick 1969).

Opinion varies as to whether this system is represented by pores which might be detectable by electron microscopy (Mayerson et al, 1960 and Coltran, 1967) or in fact takes the form of vesicular transport, as demonstrated by Jennings, Marchesi and Florey (1962), Abramson (1967), Bruns and Polade (1968) and Shea, Karnovsky and Bosset (1969). The process, "cytopemphsis", has been considered in great depth by Jennings and Florey (1967).

Likewise, it has been suggested that the small pores are in functional terms, micropinocytotic vesicles and the intercellular junctions (Coltran, 1967) as demonstrated using horse radish peroxidase (40,000 mol. wt.). Reeve and Chen (1970) are of the opinion that vesicular transport is the main pathway by which the plasma proteins enter the interstitial fluid. The mechanism is thus independent of water inflow and is able to maintain the large mass of interstitial protein in the skin and muscle, so that only slow changes occur in the interstitial C.O.P. Thus experimentation is slowly giving a more accurate picture of the role of the permeability of the capillaries.

Valuable information has also been obtained from the investigation of bulk movement of plasma proteins and their quantitation in tissues. Thus, Gitlin and Janeway (1953) showed that when the plasma was depleted there was a rapid movement (shift) of protein back into it, from the extra vascular

pool. Rothschild, Bauman, Yalow and Bersen (1954) found that the skin and muscle contain half of the total extravascular albumin whereas the liver contains less than 1%. Four to five days were needed for complete equilibration of all the exchangeable extravascular albumin in the skin and muscle. Friedman (1957) broadly confirmed the conclusions of Benson, Kim and Bollman (1955) that equilibration of albumin is rapid in the liver and the intestines, intermediate in muscle and slow in the kidney, spleen and lung. Friedman found that skin equilibration was incomplete at 12 hours. Taylor, Kinmonth, Rollinson, Rotblat and Francis (1957) using isotopically labelled plasma proteins injected subcutaneously, found no evidence of direct return to the capillaries. They concluded that the lymphatics were the most important mechanism for the transport of protein from the tissue fluids, back to the blood. Dewey, (1959), essentially found no difference between the distribution of albumin and γ globulin in the tissues (although both proteins varied considerably). Using homologous and heterologous proteins in the rat he found that 90% of the extravascular plasma proteins are present in the muscle, fat and skin. The large lymphatics represent 2 to 3 % at the most.

Our knowledge of the nature and distribution of the

extravascular proteins is limited and mostly indirect. A few workers have successfully obtained and examined samples of interstitial fluid from different tissues (Maurer, 1938, Gitlin and Janeway, 1954, Dewey, 1959, Fricke, 1961 and Creese, D'Silva and Shaw, 1962). The presence of all major plasma protein fractions has been demonstrated, although not in the same proportions. Drinker and Yoffey (1941) considered that the only controversy was with regard to the amount of protein in the interstitial fluid.

Shultze and Heremans (1966) emphasised the bound nature of the interstitial fluid. It consists of two parts, a semi-solid gel phase in which the water is firmly bound and a fluid sol phase contained in inclusion vacuoles embedded in the matrix. A major constituent of this matrix is likely to be acid mucopolysaccharides, the properties of which have been investigated in vitro by Buddeke (1960), Laurent (1966) and (1969) and Hellsuig (1969). Laurent (and Reeve and Chen, 1970) has suggested that as well as acting as ion-exchangers there will be an exclusion effect very similar to that operating in molecular sieve chromatography. Thus higher molecular weight substances which will be largely excluded, will pass through the interstitial fluid more rapidly than lower molecular weight solutes which as a result of diffusion will be present in the interstitial fluid at a higher concentration.

The quantitative significance of this exclusion in vivo, has yet to be determined. The interstial fluid appears to move as if restrained by invisible channels running alongside connective tissue fibres (Schultze and Heremans, 1966) possibly as a consequence of the exclusion effect.

The investigation of plasma protein metabolism using isotopically labelled plasma proteins and in particular the use of the isotopes of iodine, has led to the development of a number of mathematical models all of which assume extravascular distribution. Each model makes certain assumptions about one or both subdivisions of the whole body distribution, which may or may not be true under particular experimental conditions. Most models are an over-simplification but may give reasonable results, all errors involved being less than the errors involved in the quantitation itself. These models have been discussed by Freeman and Matthews (1958), Jarnum (1963), Anderson (1966), Donato (1966) and Schultze and Heremans (1966). Takeda and Reeve (1963) were unhappy about those models which represented interstitial albumin as one or more rapidly mixing compartments, which lack physiological reality. Atencio and Reeve (1965) found an increased rate of passage of fibrinogen through the interstitial fluid, compared to albumin. They concluded that this implied a different interstitial distribution.

Rothschild, Oratz and Schreiber (1970) confirmed the earlier findings of Sellers, Katz, Bonorris and Okuyama (1966) and Sellers, Katz and Bonorris (1968), that in the rat serious discrepancies are found between direct measurement of albumin distribution and calculated values from conventional models. Rothschild et al (1970) agree with the findings of Reeve and Bailey (1962) and Takeda and Reeve (1963), that the concept of a uniform homogenous extravascular pool with a single exchange rate, has no physiological reality. These findings have yet to be confirmed in other species, the techniques involved being difficult to apply to larger animals. To try and overcome some of the problems associated with conventional methods Reeve and his co-workers suggested that the extravascular compartment could be represented by a series of pipes, transporting different quantities of albumin at different rates. They (Takeda and Reeve, 1963) found that 80 to 85 % of the plasma albumin molecules will have returned to the circulation in 2 days, 93 to 97 % in 5 days, and 98 to 99 % in 10 days.

Recent reviews (Reeve and Chen, 1970, Rothschild et al 1970) confirm the findings of earlier workers that plasma protein leak occurs in all capillary beds and that the main part of the extravascular compartment is the interstitial spaces and the smallest lymphatics. Reeve et al 1970,

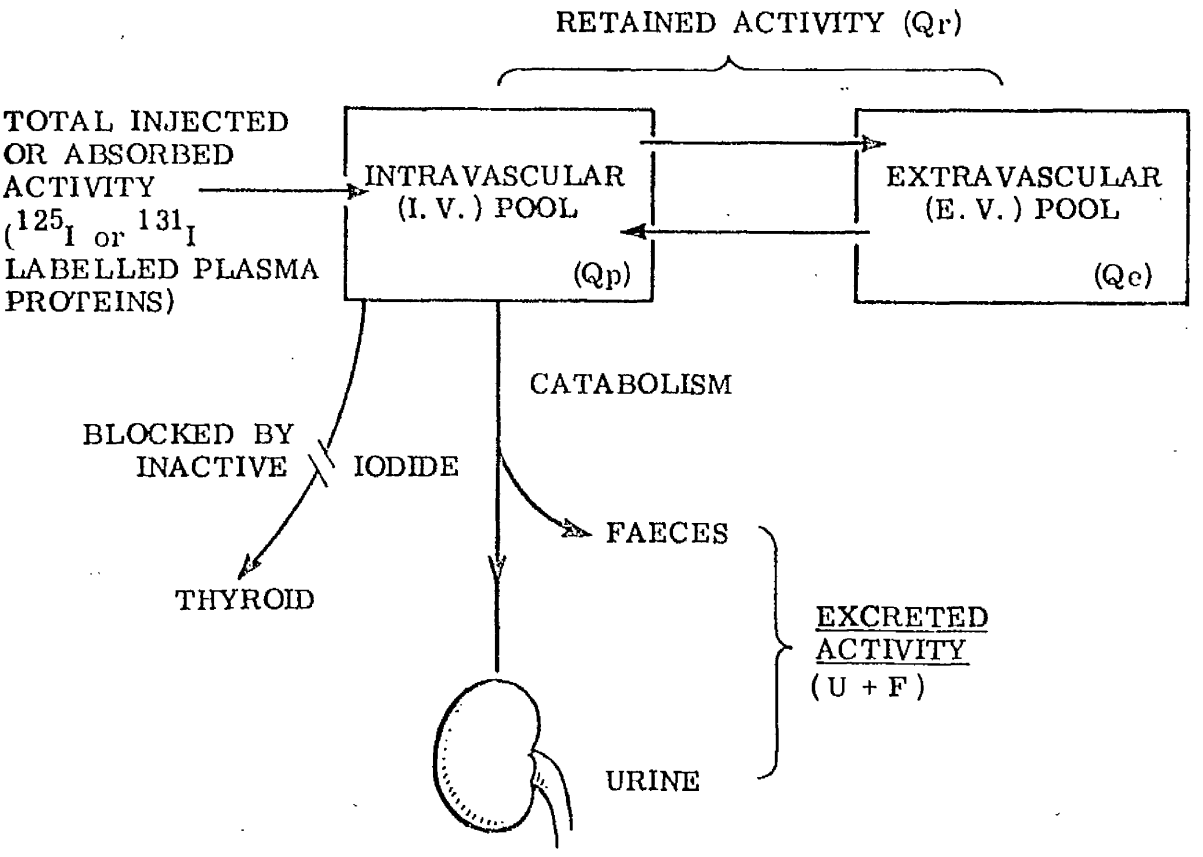
concluded that the E.V./I.V. ratio of albumin is an estimate of the ratio between the rate constants by which albumin leaves and returns to the circulation.

A considerable amount of information is thus available concerning the extravascular "circulation" of the plasma proteins. Different tissues were found to equilibrate at different rates which led up to the suggestion by Reeve and his co-workers, that the extravascular compartment (for albumin) is best represented as a series of pipes. For studies in the neonatal calf, this model and some of the more conventional ones, cannot be readily applied because of the lack of "Steady State" conditions (see Discussion in Section IV). In this situation, only the simpler models of Sterling (1951) and Campbell, Cuthbertson, Matthews and McFarlane (1956) can be used, provided their limitations are realised. Analysis of the plasma disappearance curve, as suggested by Matthews (1957) and others, from which information about transfer rates can be obtained, is thus not possible.

The "equilibrium time" method of Campbell et al (1956) (II C 8b vi) will be subsequently referred to as the Campbell method and the distribution thus obtained as the Campbell distribution. It allows for catabolism during the initial distribution period and treats the extravascular

Figure 5

THE DISTRIBUTION AND METABOLISM OF PLASMA PROTEINS IN THE NEONATAL CALF



compartment as a single protein pool (see Fig 5). The extrapolation method (II C 8a iv) of Sterling (1951) assumes instant mixing, and is thus more likely to give a false distribution. The method will be referred to as the Sterling method and the distribution thus obtained, as the Sterling distribution. Until such time as it is possible to allow for all the additional variables (e.g. by computer simulation) it will not be possible to sub-divide the extra-vascular compartment. However, in relation to the measurement of the efficiency of absorption of the colostral immunoglobulins, it is not necessary to take account of any slowly exchanging parts of this compartment (see Section VI - Discussion). The Campbell method thus becomes acceptable and the Sterling method was also used, as it does not require the collection of urine and faeces, and is thus much more easily applied. If the results obtained by both methods show a close agreement, it may be possible, in future studies, to use the Sterling method alone.

An essential requirement for a study of this kind is a sufficient quantity of pure IgG_1, (see Section III, for the method of preparation) for labelling with the isotopes of iodine. The concept of purity with regard to proteins has developed as new techniques have been applied. Thus when

Pirie (1940) examined the problems of purity in relation to large molecules, after discussing the various criteria in current use, he concluded that purity was a concept which has no meaning except with reference to the methods used and assumptions made. These methods were further examined by Shedlovsky (1943) and Chow (1944). Edsall (1947) was critical of those workers who considered the method of preparation as sufficient evidence of purity.

Colvin, Smith and Cook (1954) suggested that a preparation may be considered pure for a given purpose and should be regarded as a population of more or less closely related molecules. Further support for a relative concept of purity came from Steinberg and Mihalyi (1957) who also warned against artifacts resulting in false conclusions. Vaughan and Steinberg (1959) recognised that the best that could be hoped for was the accumulation of evidence of the absence of impurities which increases the probability that a protein is in fact pure. Thus available methods could only establish homogeneity with respect to certain physical properties.

The development of immunochemical methods, in particular Double Diffusion and Immunoelectrophoresis has provided very sensitive methods for the detection of contaminants in protein preparations. (Kabat and Meyer, 1961, Lontie, 1961, Freeman, 1970

and Grant and Butt, 1970). Andersen (1966) used several methods, bases on different physical properties to ensure a high probality that his IgG preparation was pure. Once it is established by several criteria that a preparation is sufficiently pure, the other question which must be asked is whether or not it is denatured (Freeman, 1970). Part of the answer is provided by the tests for purity i.e. that the preparation is uniform. This subsequently arose as a particular problem in relation to Bovine IgM (see Section V).

For the purpose of these studies, Electrophoresis, Immunoelectrophoresis, Double Diffusion and after labelling, Rechromatography, were chosen for determining the suitability of the IgG_1 preparations for the metabolic studies. Only a trace impurity, detected by Immunoelectrophoresis and Double Diffusion (see Section III B and C) was accepted, the error involved being less than the errors involved in the metabolic study.

In spite of the overwhelming evidence for the extravascular circulation of plasma proteins reviewed above, it has still been possible for some workers to ignore it or forget about it. Thus Pierce and Smith (1967) tried to quantitate absorption in the neonatal piglet using plasma levels. Klaus Bennet and Jones (1969) using radial diffusion claimed to quantitate the absorption of bovine colostral IgG and IgM, although they completely ommitted the extravascular distribution of these immunoglobulins.

Balfour and Comline (1962) avoided the problem in their study of factors accelerating absorption of colostrum by collecting thoracic duct lymph over a 300 minute period. During this time, they recovered 12 to 25 % of their labelled globulin. The calves were anaesthetised throughout the experiment.

Other workers while recognising the importance of taking the size of the extravascular pool into account, were forced to estimate its size, on the basis of figures available at the time. Thus McEwan (1968) used the Extravascular/Intravascular (E.V./I.V.) ratio of 0.7/1 (Nansen and Nielsen, 1966 and Nielsen and Nansen, 1967) for Slow IgG (IgG_2) in adult cattle. Kruse (1969) on the other hand, applied data from work with Human IgG.

Selman (1969) confined himself to comparisons of plasma levels of immunoglobulins, thus avoiding bringing the extravascular size into his calculations. McEwan (1968) and Selman (1969) assumed that distribution between intra and extravascular compartments would be complete by 48 hours after the ingestion of colostrum.

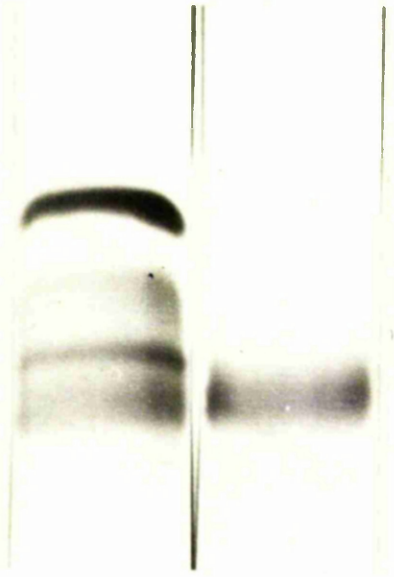
Therefore, before quantitating the efficiency of absorption of the colostral immunoglobulin in the neonatal calf, it was necessary to know more about their metabolism and in particular the distribution between intra and extra-

vascular compartments, the rate of equilibration between the compartments and the rate of degradation of the immunoglobulins (Anderson 1964, Macdougall and Mulligan, 1969). Of the immunoglobulins present in bovine colostrum, Fast IgG (IgG_1) is present in the highest concentration. Its metabolism was therefore studied first.

Figure 6

Examination of Fast IgG, prior to Isotopic Labelling

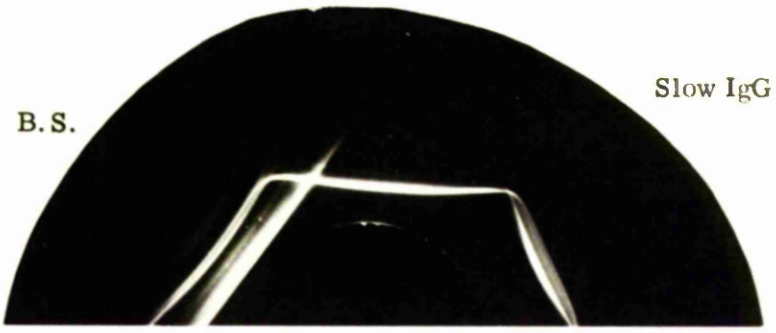
1. Electrophoresis



B.S. Fast IgG

2. Double Diffusion

Fast IgG



A. B. G.

3. Immunoelectrophoresis

Fast IgG

A. B. G.

B.S.

B.S. - Bovine Serum

A. B. G. - Anti-bovine-globulin (with some anti-albumin activity)

MATERIALS AND METHODS

A. Source of Calves

Calves 1, 4, 5, 6 and 7 were supplied by Dr. I. Selman, Department of Veterinary Medicine, University of Glasgow. They were all 48 hours old. Calves A to H were market calves, purchased through a dealer. They were up to one week old. Calves 8 to 14 were purchased from Mr. H. Guthie, Moss Side Farm, Kilmarnock. They were 2 to 5 days old.

B. Examination of the IgG_1 preparation - Fig. 6.

(see Section III for the method of preparation)

1. Cellulose Acetate Electrophoresis

(see Section II D 3a)

The preparations gave a single broad band on electrophosesis, in the $\beta\gamma$ region.

2. Double Diffusion

One main precipitin line developed, which showed complete identity with one of the lines in the reference serum.

3. Immunoelectrophoresis

(see Section II E 3)

Immunoelectrophoresis of the final IgG preparations was carried out using Anti-Bovine Serum and Anti-Bovine Globulin. They were run opposite whole adult bovine serum and a purified Slow IgG (IgG_2) preparation, for comparison. Each preparation showed the characteristic arc of Fast IgG (see Fig. 3).

Some of the preparations (see Section III C 4) showed slight traces of a contaminant by Immunoelectrophoresis and / or Double Diffusion. This was possibly Bovine IgA. The degree of contamination was considered to be low enough to be disregarded for the metabolic study.

4. Anti-Trypsin Activity

The method used was that of Kueppers and Bearn (1966) as described by Shim, Kang, Kim, Cho and Lee (1969) for the examination of human colostral IgA. Purified preparations of 2% IgG_1, from colostral whey and serum were compared against whey and serum respectively. While both whey and serum showed marked anti-tryptic activity, neither of the IgG_1 preparations showed any.

C. Bovine Albumin

A 2% solution of commercial bovine albumin (Cohn Fr V - Armour, Eastbourne, Sussex) was made up for labelling. It was examined by Electrophoresis and after labelling, by molecular sieve chromatography on Sephadex G-200 (see Fig. 12).

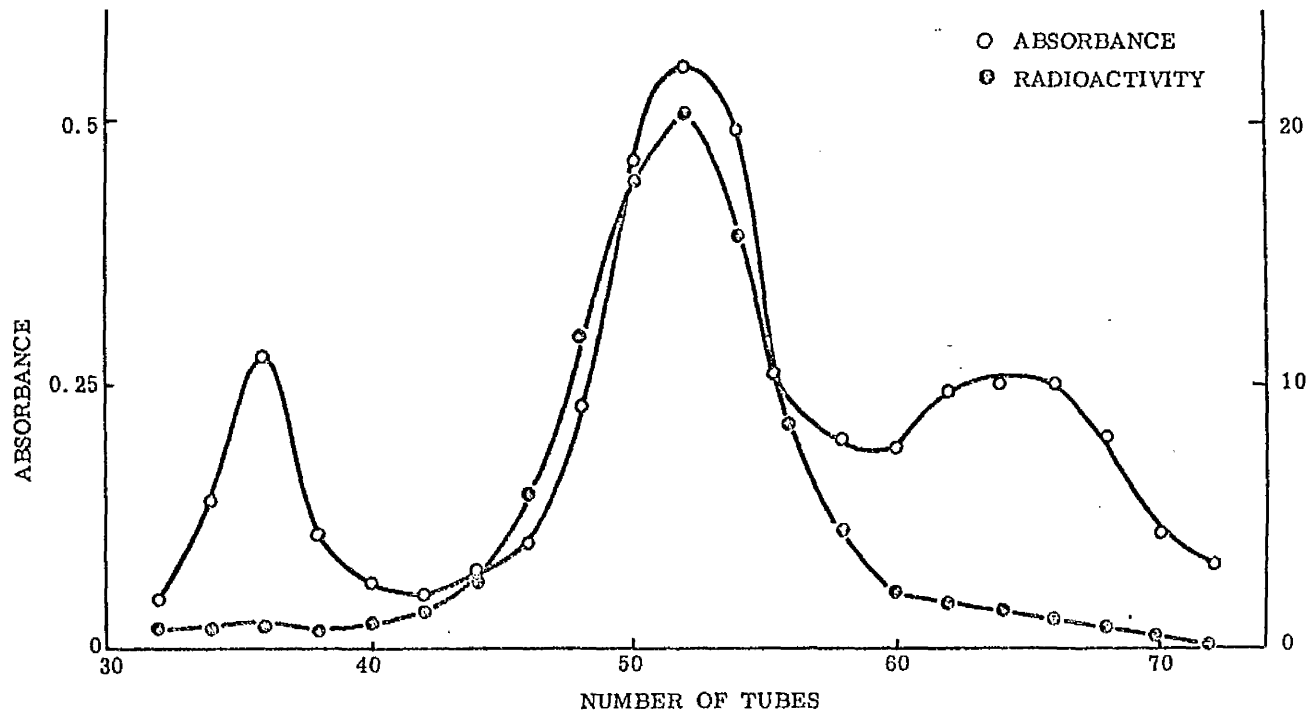
D. Labelling

(see Section II C 1a)

As the experiments were spread over a period of 10 months, IgG preparations were labelled as required. Less

Figure 7

CHROMATOGRAM OF CALF SERUM CONTAINING ^{125}I-LABELLED IgG_1, ON SEPHADEX G200



than 1.5 atoms of iodine were incorporated per molecule of IgG_1. Similarly, less than 1.0 atoms I/molecule of albumin were incorporated (see Dargie, 1969, for the method of calculation). More than 95% of the radioactivity in the final IgG_1 and albumin preparations, was protein bound.

E. Rechromatography of Labelled IgG

0.1 ml of the labelled preparation was added to 4 ml of calf serum and applied to a Sephadex G-200 column (see Section III B) for molecular sieve chromatography. The radioactivity was determined in the 4 ml fractions and plotted to show its relationship to the protein concentration. (See Fig. 7). The radioactivity was always found to be associated with the middle peak of the chromatogram.

F. Injection

The experimental animals were divided into 3 groups.

Group 1 consisted of 12 calves, for the initial IgG_1 metabolic studies.

Group 2 consisted of 4 calves, for repeat determinations of distribution and catabolism of IgG_1. An additional calf from Group 1 was also included.

Group 3 consisted of 7 calves for the study of Albumin metabolism, to provide a comparison for the IgG_1 results.

The calves in Groups 1 and 2 each received 2 to 5 mc

of ^{125}I or ^{131}I labelled IgG_1. The calves in Group 3 each received approximately 1.5 mc of ^{131}I - labelled bovine albumin. When injected, all calves were at least 72 hours old.

G. Metabolic Studies

These were carried out for 14 days, during which time the calves were kept in metabolic cages.

H. Further Experimental Details

See Section II C 1 to 7 .

I. Calculation of Results

See Section II C 8 .

J. Diarrhoeic Calves

Calves were considered to be diarrhoeic when they produced faeces corresponding to the ++ and / or +++ classification of de la Fuente, 1970.

Figure 8

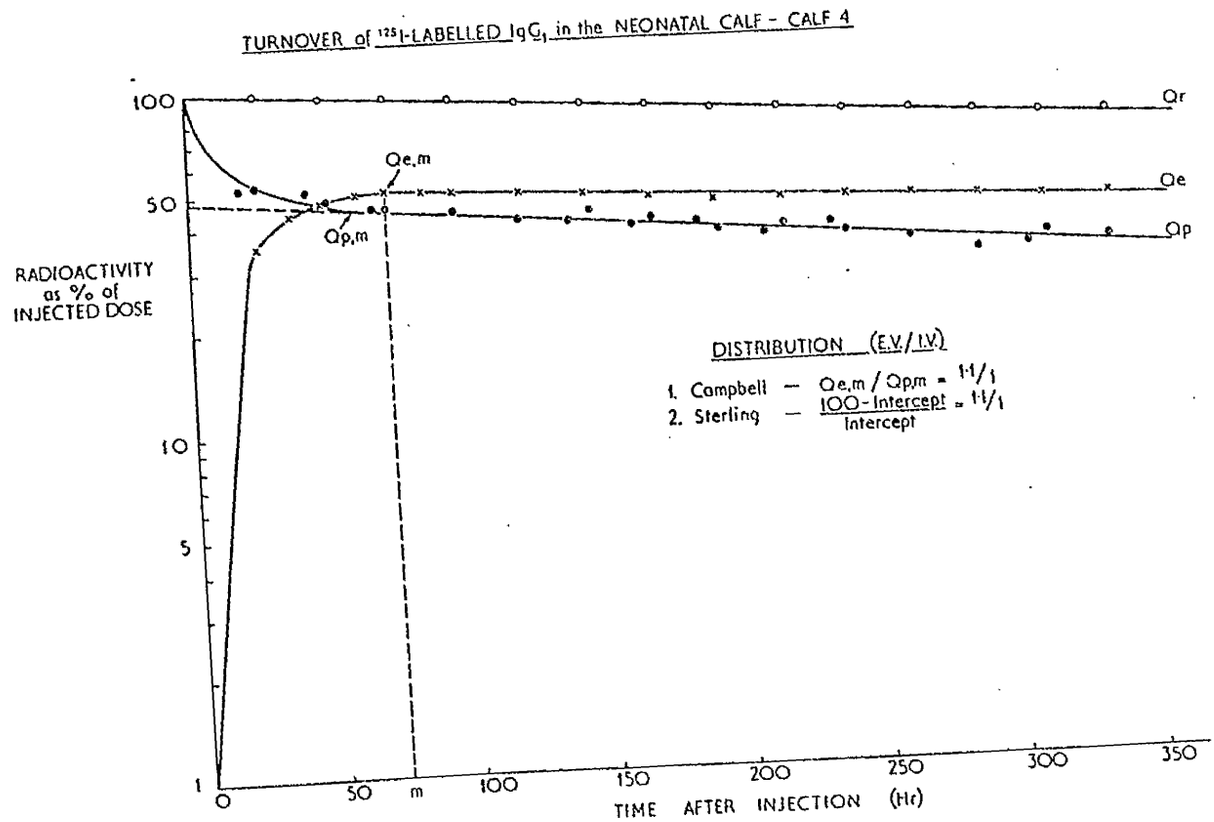

TURNOVER of ^{125}I-LABELLED IgG$_1$ in the NEONATAL CALF - CALF 4
100
50
10
5
1
RADIOACTIVITY as % of INJECTED DOSE
Qr
Qe,m
Qe
Qp,m
Qp
DISTRIBUTION (E.V./I.V.)
1. Campbell — Qe,m / Qp,m = 1·1/1
2. Sterling — 100 - Intercept / Intercept = 1·1/1
0
50
m
100
150
200
250
300
350
TIME AFTER INJECTION (Hr)

Table 2

The Distribution (by Campbell and Sterling methods) And the Catabolism of Labelled IgG_1 in 12 Calves - Group 1

Calf No.	E.V./I.V. Sterling Ratio	E.V./I.V. Campbell Ratio	Apparent Equilibrium Time (hours)	Total Body Catabolic Rate	Fractional Catabolic Rate
1(♀)	1.2/1	1.2/1	60	0.026	0.063
4	1.1/1	1.1/1	60	0.023	0.051
5	1.4/1	1.4/1	60	0.029	0.067
6	1.2/1	1.1/1	46	0.040	0.082
7	1.3/1	1.2/1	43	0.056	0.130
E	1.7/1	1.5/1	36	0.044	0.092
F	1.4/1	1.2/1	36	0.055	0.099
G	1.2/1	1.3/1	36	0.052	0.100
H	1.3/1	1.2/1	8	0.042	0.083
12	1.9/1	1.3/1	60	0.050	0.099
13	1.6/1	1.3/1	60	0.041	0.084
14	1.7/1	1.2/1	36	0.055	0.112
Mean	1.5/1	1.3/1	45	0.043	0.089
S.D.*	0.25/	0.11/	16	0.012	0.022
S.E.*	0.07/	0.03/	4.6	0.003	0.006

RESULTS

A. Group 1 - IgG_1 Metabolism

(Tables 2, 3 and 4)

The distribution was calculated by two methods (see Fig. 8). The equilibrium time method of Campbell et al (1956) gave a mean Extravascular/Intravascular (E.V./I.V.) ratio of 1.3 ± 0.11/1. The extrapolation method of Sterling (1951) gave a similar distribution of 1.4 ± 0.25/1 (E.V./I.V.) . There was a significant positive correlation between the two methods at the 5% level (Fig. 9).

The apparent equilibrium time was 45 ± 16 hours. 8.9 ± 2.2 % of the plasma pool was catabolised per day, and 4.3 ± 1.2 % of the Total Body pool. The apparent plasma and total body $T_{\frac{1}{2}}$'s were 16.1 ± 2.7 and 18.4 ± 5.7 (days) respectively. Very significant positive correlations were found between the Campbell distribution and all four of the above measures of catabolism (see Fig. 10). Similar correlations were found between the F.C.R. and the Total Body C.R. (Fig. 11) and between the Plasma and Total Body $T_{\frac{1}{2}}$'s ($r = 0.874$, $p = <0.001$).

The plasma volumes at the start of the experiment were 63 ± 12 ml/Kg. Changes in body weight during the experimental period varied considerably, 5 ± 14 %, ranging from -12 up

Table 3

Half - Lives and additional data for Calves in Group 1

Calf No.	Plasma Volume (ml/Kg)	Apparent Plasma $T_{\frac{1}{2}}$ (days)	Total Body $T_{\frac{1}{2}}$ (days)	% Change in P.V. (14days)	% Change in Body Weight (14days)
1	66	17.8	26.0	-	5
4	84	20.0	30.0	-	28
5	76	17.0	24.6	-	23
6	60	16.7	17.7	-	-11
7	49	11.6	12.3	-	-12
E	66	15.5	17.1	-	18
F*	63	17.0	12.7		
G	79	10.5	13.2	5	10
H	60	15.1	17.9	7	14
12	45	17.6	16.7	2	-7
13	59	16.7	18.7	-7	-8
14	56	18.1	13.6	-13	-8
Mean	63	16.1	18.4	-1.3	5
S.D.±	11.6	2.7	5.7	8	15
S.E.±	3.4	0.8	1.7	4	4

* Died Day 12

Table 4

Plasma Protein Results (gm/100ml) and Mean P.C.V. for Group 1 Calves

Calf No.	Mean P.C.V.	Day 0		Day 7		Day 14	
		Total Protein	A/G Ratio	Total Protein	A/G Ratio	Total Protein	A/G Ratio
1	35	5.1	0.55			5.3	0.62
4	29	4.1	0.47	3.7	0.80	4.0	0.65
5	30	4.4	0.42	3.8	0.56	4.2	0.60
6	31	5.1	0.52	4.9	0.54	5.1	0.59
7	44	6.3	0.46	6.3	0.38	5.5	0.43
E	28	5.7	0.44	5.6	0.57	5.6	0.48
F	33	3.6	0.65	3.1	0.70		
G	28	5.7	0.42	5.5	0.53	5.6	0.46
H	37	5.0	0.43	4.7	0.56	5.0	0.60
12	48	5.5	0.57	5.6	0.56	5.7	0.69
13	36	4.2	0.80	4.6	0.74	4.8	0.68
14	50	4.6	0.68	4.7	0.77	4.9	0.66
Mean	36	4.9	0.53	4.8	0.63	5.1	0.59
S.D.±	7.7	0.78	0.12	0.96	0.13	0.57	0.08
S.E.±	2.2	0.23	0.03	0.29	0.04	0.17	0.03

Figure 9

THE CORRELATION BETWEEN THE CAMPBELL AND STERLING DISTRIBUTIONS (BOTH EXPRESSED AS THE RELATIVE SIZE OF THE EXTRAVASCULAR COMPARTMENT) OF IgG_1 IN 12 CALVES (GROUP 1)

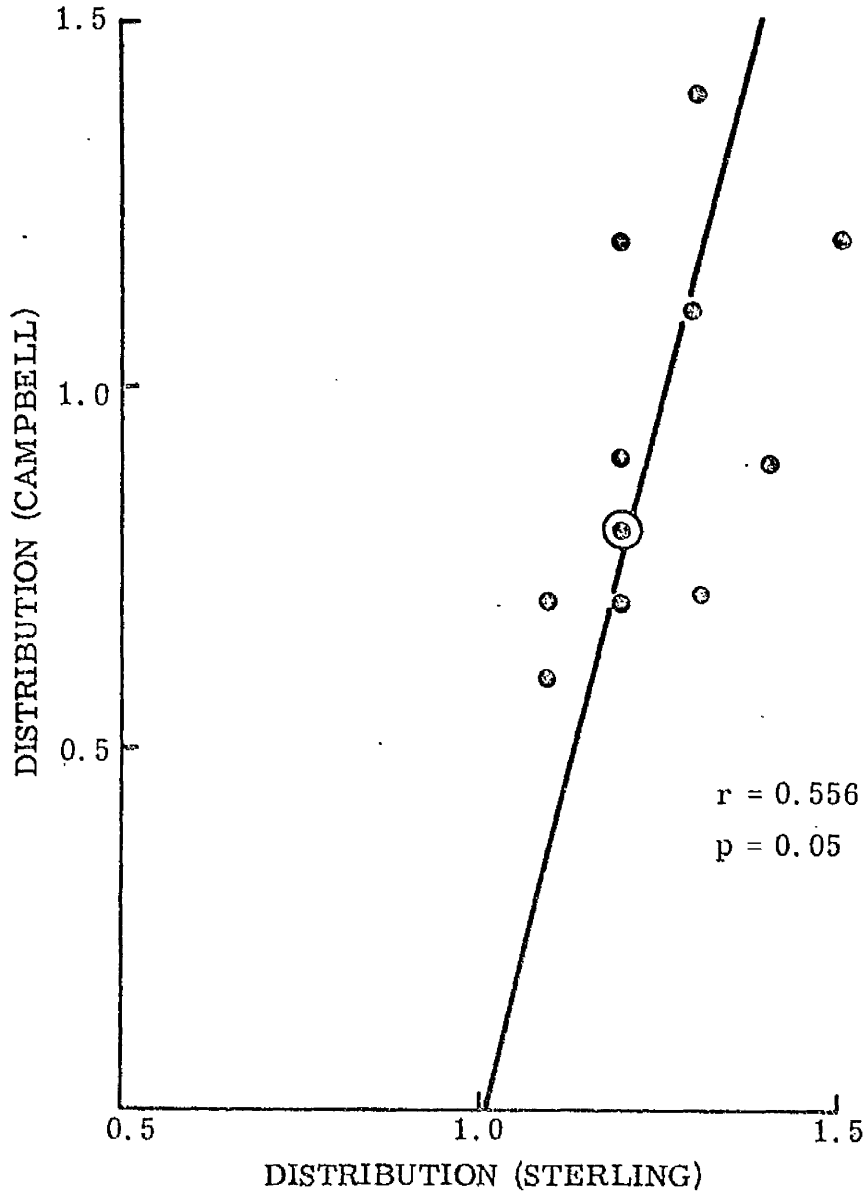


Figure 10

CORRELATIONS BETWEEN THE CAMPBELL DISTRIBUTION (EXPRESSED AS THE RELATIVE SIZE OF THE EXTRAVASCULAR COMPARTMENT) AND THE FOUR MEASURES OF CATABOLISM

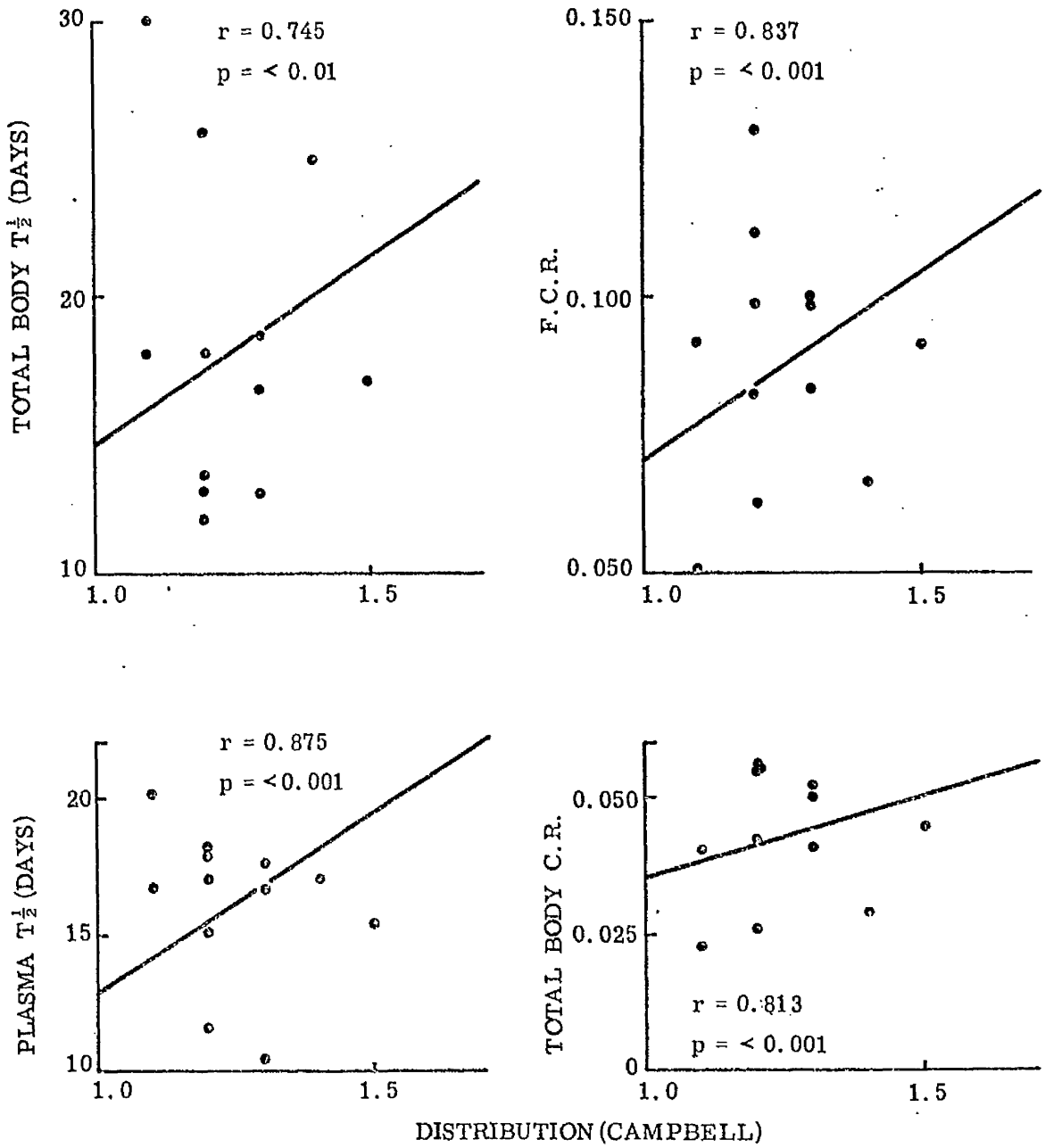
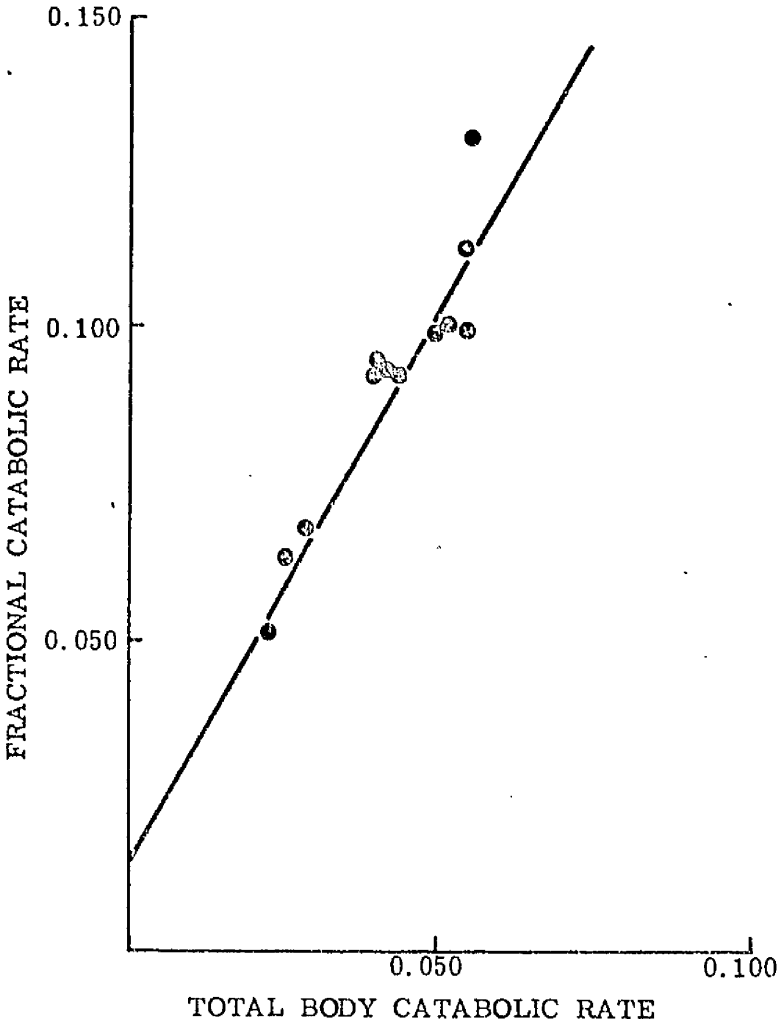


Figure 11

THE CORRELATION BETWEEN THE FRACTIONAL CATABOLIC RATE AND THE TOTAL BODY CATABOLIC RATE OF IgG_1, IN 12 CALVES (GROUP 1)



to +28%. No significant correlation was found between the Campbell distribution and the Plasma Volume (ml/Kg). The calves had a mean P.C.V. of 36 ± 8. The plasma protein changes are shown in Table 4.

The three calves (1, 4 and 5) which did not develop diarrhoea, had an E.V./I.V. ratio of 1.2/1, (Campbell and Sterling), an apparent equilibrium time of 60 hours, an F.C.R. of 6.0% of the intravascular pool/24 hours, a Total Body Catabolic Rate of 2.9%/24 hours, an apparent plasma $T\frac{1}{2}$ of 18 days and a Total Body $T\frac{1}{2}$ of 27 days (all expressed as the mean value).

The following additional information was also obtained from Calves E to H, with regard to the appearance of radioactivity in the urine. Calves E and F first passed urine within 2 to 3 hours after injection (three times in the first 11 hours). Calf G passed urine only once between 8 and 11 hours post injection. Calf H first passed urine within 2 hours. (twice in 11 hours). All the urine samples collected contained significant levels of radioactivity, when counted. There was thus considerable variation, in both the interval between injection and the first urination and also in the frequency of urination.

Table 5

Repeat Determinations of the Distribution and Catabolism of Labelled IgG_1 in Four Calves - Group - 2

Calf No.	Age (weeks)	E.V./I.V. Sterling Ratio	E.V./I.V. Campbell Ratio	Total Body Catabolic Rate	Fractional Catabolic Rate
8(1)	1	1.4/1	1.0/1	0.071	0.014
9(1)♀	1	1.1/1	1.1/1	0.045	0.085
10(1)	1	1.3/1	1.4/1	0.051	0.123
11(1)	1	1.9/1	1.7/1	0.042	0.120
8(2)	4	1.5/1			
9(2)♀	4	1.2/1	1.1/1	0.020	0.045
10(2)	4	0.89/1	0.87/1	0.049	0.086
11(2)	4	1.04/1	1.0/1	0.043	0.084
13(2)	12	0.92/1	0.98/1	0.044	0.097
9(3)♀	15	0.96/1	0.64/1	0.047	0.089

B. Group 2 - Repeat Determinations

A significant negative correlation ($r = -0.66$, $p = \leq 0.05$) was found between the age of the calves and the Campbell distribution. i.e. as the age of the calves increased, the relative size of the extravascular compartment decreased. The negative correlation with the Sterling distribution was not significant. Although the difference between the calves at 1 and 4 weeks was not significant for Campbell or Sterling distributions, calves 10 and 11 had distributions below the range of 1.1 - 1.5/1 (Campbell) and 1.1 - 1.9/1 (Sterling) found in Group 1. A highly significant positive correlation ($r = 0.875$, $p = 0.001$) was found between the F.C.R. and the total body catabolic rate. No significant correlation was found between the F.C.R. and the age of the calves, possibly reflecting the influence of diarrhoea in the initial study, at 1 week of age. However, when the F.C.R. s at 1 week were replaced by those obtained for the non-diarrhoeic calves (1, 4 and 5 in Group 1, see Table 2), a significant positive correlation was found between the age of the calves and the Fractional Catabolic Rate ($p = 0.05$).

Figure 12

CHROMATOGRAM OF CALF SERUM CONTAINING ^{131}I-LABELLED BOVINE ALBUMIN ON SEPHADEX G200

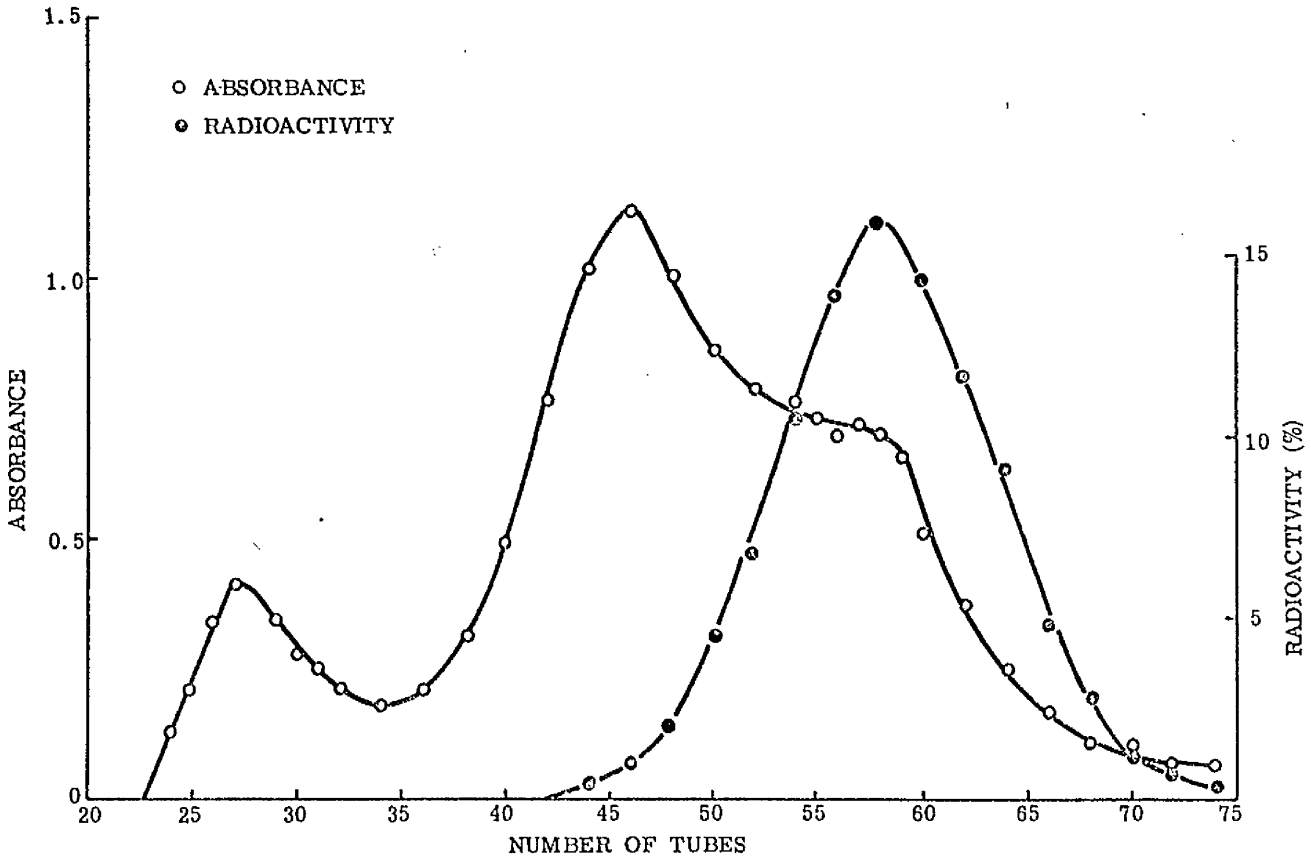


Table 6

The Distribution and Catabolism of Labelled Albumin in 7 Calves - Group 3

Calf No.	E.V./I.V. Sterling Ratio	E.V./I.V. Campbell Ratio	Apparent Equilibrium Time (hours)	Total Body Catabolic Rate	Fractional Catabolic Rate
A	1.6/1	1.5/1	36	0.024	0.084
B	1.9/1	1.9/1	36	0.016	0.061
C	1.9/1	1.8/1	36	0.021	0.090
D	1.9/1	2.0/1	36	0.030	0.120
12	2.4/1	2.2/1	72	0.029	0.116
13	1.7/1	1.7/1	84	0.026	0.077
14	2.0/1	1.8/1	48	0.041	0.109
Mean	1.9/1	1.9/1	50	0.027	0.094
S.D.±	0.25/	0.22/	20	0.008	0.022
S.E.±	0.09/	0.08/	8	0.003	0.008

C. Group 3 - Albumin Catabolism

The same parameters were determined (as for Group 1). The Campbell and Sterling methods both gave an E.V./I.V. ratio of 1.9/1. A highly significant positive correlation was thus found between them ($r = 0.928$, $p = 0.001$). The apparent equilibrium time was 50 (± 20) hours. 9.4 (± 2.2) % of the plasma pool was catabolised per day and 2.7 (± 0.8) % of the total body pool. The apparent plasma and total body half-lives were 19.2 (± 4.6) and 23.8 (± 3.9) days respectively.

The plasma volumes at the start of the experiment were 60 ± 6 ml/Kg. A highly significant negative correlation was found between plasma volume and the E.V./I.V. (Campbell) ratio ($p = <0.001$) i.e. the larger the relative size of the extravascular compartment, the lower the plasma volume (ml/Kg) see Fig 13. Positive correlations found were between the total body half-life and the total body catabolic rate ($r = 0.663$, $p = <0.05$). The correlation between the total body half-life and the fractional catabolic rate was not significant at the 5% level. Significant correlations were not found between the E.V./I.V. ratio and the four measures of catabolism. The positive correlation between the % P.V. change and the % body weight change was significant. ($r = 0.918$, $p = <0.01$).

Figure 13

THE CORRELATION BETWEEN THE ALBUMIN DISTRIBUTION (CAMPBELL) EXPRESSED AS THE RELATIVE SIZE OF THE EXTRAVASCULAR COMPARTMENT AND THE PLASMA VOLUME (ml/Kg)

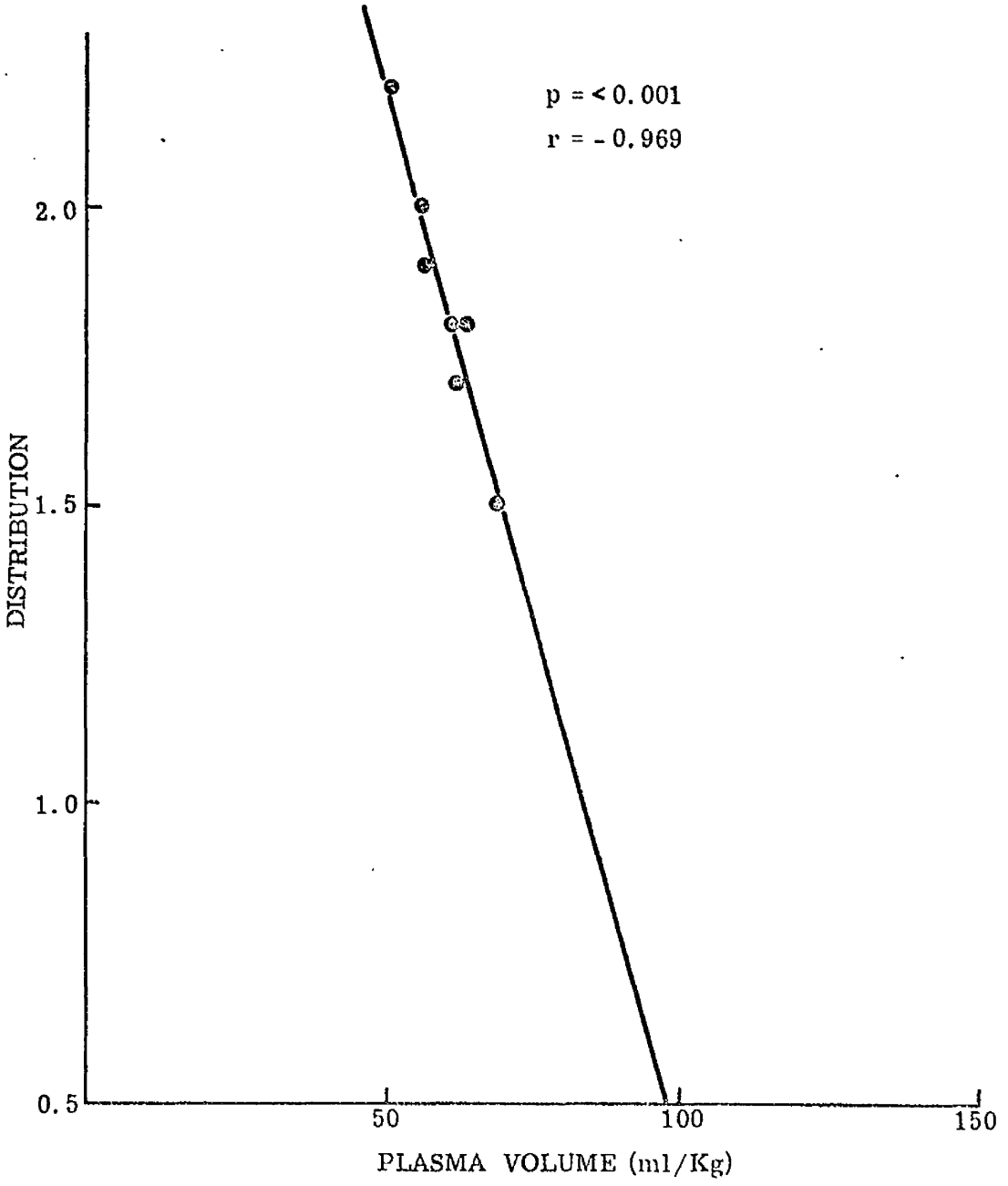


Table 7

Half - Lives and additional data for Calves in Group 3

Calf No.	Plasma Volume (ml/Kg)	Apparent Plasma $T_{\frac{1}{2}}$ (days)	Total Body $T_{\frac{1}{2}}$ (days)	% P.V. Change in 14 days	% Body Weight change in 14 days
A	69	18.5	27.8	3	7
B	57	25.2	27.6	25	20
C	64	24.8	21.4	14	16
D	56	15.4	21.3	10	10
12	50	18.7	24.7	2	-7
13	62	12.7	26.4	-7	-8
14	61	18.9	17.2	-13	-8
Mean	60	19.2	23.8	5	4
S.D.±	6.1	4.6	3.9	13	12
S.E.±	2.3	1.7	1.5	5	5

Table 8

Plasma Protein Results (gm/100ml) and Mean P.C.V. for Group 3 Calves

Calf No.	Mean P.C.V.	Day 1 Total Protein	Day 1 A/G Ratio	Day 7 Total Protein	Day 7 A/G Ratio	Day 14 Total Protein	Day 14 A/G Ratio
A	34	3.6	0.56	3.4	0.56	3.3	0.68
B	39	3.8	0.66	3.9	0.75	3.6	0.83
C	35	6.6	0.31	6.0	0.40	5.8	0.44
D	41	5.0	0.42	5.1	0.46	5.3	0.47
12	48	5.5	0.57	5.6	0.56	5.7	0.69
13	36	4.2	0.80	4.6	0.74	4.8	0.68
14	50	4.6	0.68	4.7	0.77	4.9	0.66
Mean	40	4.8	0.57	4.8	0.61	4.77	0.63
S.D.±	6.3	1.0	0.16	0.91	0.15	0.98	0.10
S.E.±	2.4	0.4	0.06	0.34	0.06	0.37	0.00

D. The Effect of Diarrhoea

A number of workers (see Introduction, Section VII) have demonstrated the hypercatabolism of plasma proteins associated with diseases of the alimentary tract, particularly those causing diarrhoea.

1. Albumin

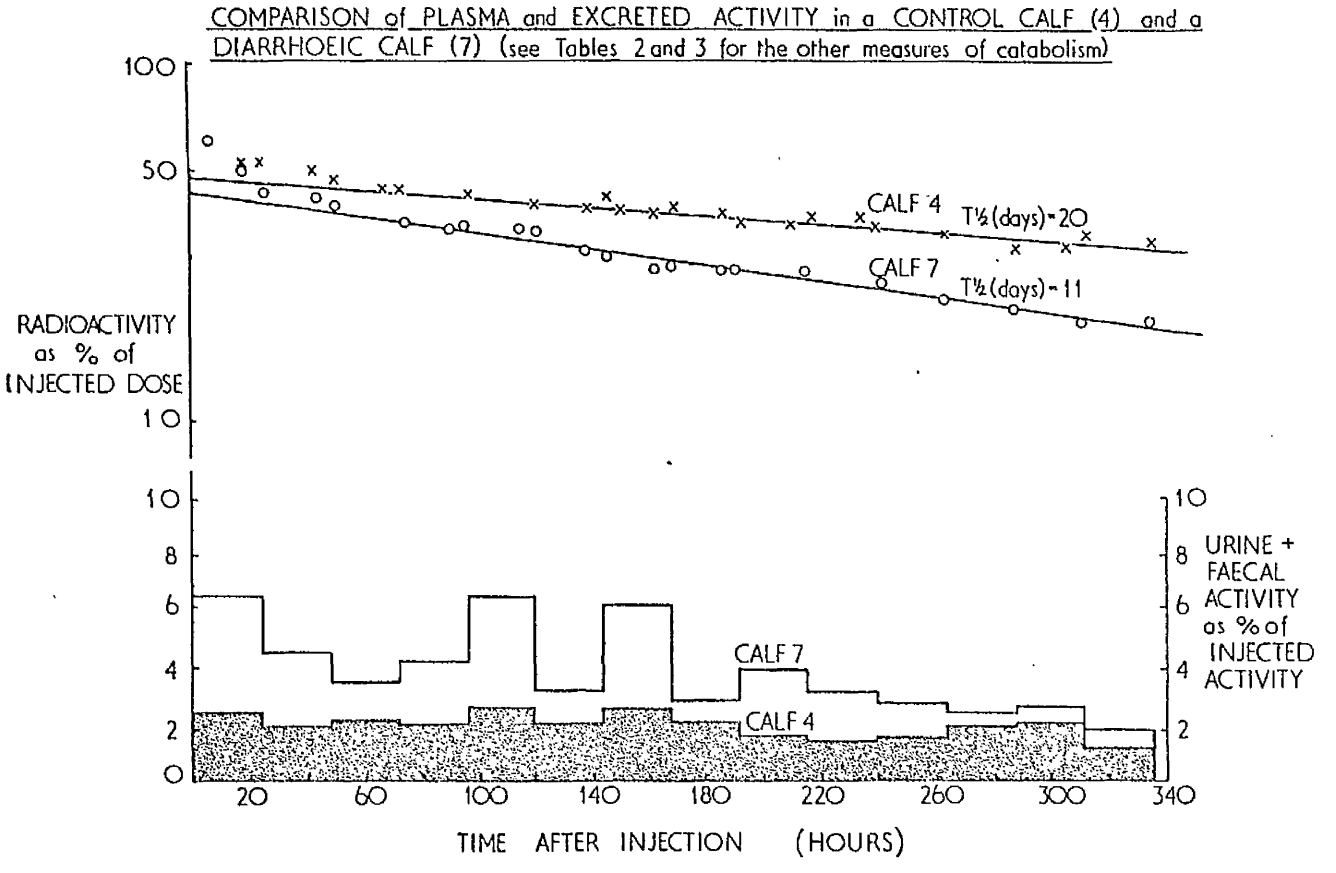
In Group III there did not appear to be any relationship between the severity and duration of the diarrhoea and the catabolism of albumin. As none of the calves remained non-diarrhoeic during the experimental period it is not possible to come to any definite conclusions as to the influence of diarrhoea on the catabolism of albumin in the neonatal calf.

However it is interesting to note that the mean, fractional catabolic rate, and plasma half lives of the seven calves were close to the control values Nielsen (1966) obtained in adult cattle.

2. IgG_1

A comparison was made between the three calves 1, 4 and 5 which did not develop diarrhoea and the remaining calves in Group 1 , along with four calves from Group 2 (at one week). The "t" test showed significant differences between the two groups, for the following parameters;

Figure 14


COMPARISON of PLASMA and EXCRETED ACTIVITY in a CONTROL CALF (4) and a DIARRHOEIC CALF (7) (see Tables 2 and 3 for the other measures of catabolism)
100
50
10
RADIOACTIVITY as % of INJECTED DOSE
CALF 4 T½(days)=20
CALF 7 T½(days)=11
10
8
6
4
2
0
CALF 7
CALF 4
URINE + FAECAL ACTIVITY as % of INJECTED ACTIVITY
20
60
100
140
180
220
260
300
340
TIME AFTER INJECTION (HOURS)

Fractional Catabolic Rate $p = <0.01$

Total Body Catabolic Rate $p = <0.001$

Total Body Half-Life $p = <0.001$

Significant differences were not found for the distribution (by both methods) and the apparent plasma half-life (the least satisfactory measure of catabolism).

Figure 14 shows a comparison between a control and a diarrhoeic calf.

DISCUSSION

Other workers have confined their investigation of the metabolism of albumin and IgG in the bovine, to studies in more mature animals. Thus Nielsen (1966a) determined the distribution of Albumin in 6 five year old Jersey cows. Including one repeat determination, he obtained a mean distribution of 1.22 ± 0.24/1 (E.V./I.V.) by an equilibrium time method. These animals had a plasma half life of 20.4 ± 3.8 days and a fractional catabolic rate of 7.9% ± 1.1. Halliday, Mulligan and Dalton (1968) obtained similar values for their 7 control animals i.e. a plasma half life of 24.0 ± 2.6 days and a fractional catabolic rate of 6.7% ± 0.8 . Holmes (1970) found a lower E.V./I.V. ratio than Neilsen, of 0.82 ± 0.07/1 (E.V./I.V.) in 5 yearlings, using the Sterling method. The plasma half lives and fractional catabolic rates of these animals were 21.5 ± 0.2 days and 6.9% ± 0.5 respectively. The distribution (E.V./I.V.) of 1.9/1 for albumin (see Table 6) in the neonatal calf, thus represents a proportionately larger extravascular pool. The plasma half lives and fractional catabolic rates are however of a similar order.

The only other information about the metabolism of Fast IgG in healthy cattle has been obtained by Nansen (1970)

in one cow (3½ years old) used to compare Slow and Fast IgG. He found an identical distribution of 0.61/1 for Slow and 0.62/1 for Fast IgG, but different plasma half-lives and catabolic rates of 18.5/7.9 days and 6.0%/14.1% (Slow/Fast respectively). In the same study in 14 cattle (average age 3 years) he found a distribution of 0.65/1 for Slow IgG, a Plasma Half-Life of 17.6 days and a Fractional Catabolic rate of 6.6%. These results were similar to those from an earlier study using the Matthews method of calculation, which gave an E.V./I.V. distribution of 0.69/1 in 6 animals. Holmes (1971) using Fast IgG in sheep obtained a very similar distribution of 1.0/1 to that previously found for Slow IgG of 1.1/1 (Holmes, 1969). It can therefore be concluded that in the adult ruminant, Fast and Slow IgG have the same distribution, as would be expected on the basis of their molecular weights.

When these results for Slow and Fast IgG in older animals, are compared with the results (in this section) for the neonatal calf, it is clear that there is a proportionately larger extravascular pool, i.e. an E.V./I.V. ratio (see Table 2) of 1.3/1 compared to Nansons ratio of 0.65/1 for Slow IgG. For the three non-diarrhoeic calves, the ratio was 1.2/1. The fractional catabolic rates and plasma half-lives of these calves were similar to Nansons control values.

Also, the mean F.C.R. for all 12 calves, of 8.9% is lower than the 14.1% found by Nansen for Fast IgG_1 in one cow.

There is evidence that this alteration in the relative size of the extravascular pool, found in the neonatal calf also occurs in man. In a group of healthy men and women, 16 to 58 years old, Cohen, Freeman and McFarlane (1961) obtained the following distributions:

Distribution (E.V./I.V.)	Number of estimations	Method of calculation
1.4 ± 0.17/1	6	Sterling
1.07 ± 0.10/1	4	Campbell
1.30 ± 0.14/1	6	Matthews

Combining the results from the three methods, we obtain a mean distribution of 1.3/1 , which corresponds to that obtained by the Matthews method.

Krasilnikoff, Anderson and Rossing (1966) obtained an E.V./I.V. ratio of 2.0/1 in two babies, 13 and 18 days old. From this age, up to 3 years, they found a gradual change in distribution, but from 3 to 8 years it remained fairly constant at 1/0.96 . Using their data for the distributions of the six children from 13 days to 3 years, a significant negative

correlation between albumin distribution and age is found ($p = < 0.01$). From these results we find a decrease in the percentage Extravascular from 67% to 50% i.e. a fall of 17%. If we replace the ratio of 1/1 for the oldest children with that obtained by Cohen et al (1961) for adults we find a fall of 10%.

A similar comparison of the bovine albumin results gives a decrease from 66% to 44% (in yearlings) and an overall decrease to 55% i.e. a fall of 11%. For IgG_1, the decrease is from 55 to 41% i.e. a fall of 14%, when we compare the distributions of calves 1, 4 and 5 (Table 2) with Nansens results. Thus, it can be seen from the changes in albumin distribution in man, associated with age and the similar changes for both albumin and IgG_1 in the neonatal calf, that in each case there is a similar age related decrease in the relative size of the extravascular compartment. This occurs even though albumin is being synthesised in the neonatal calf whereas IgG is not.

A larger extravascular compartment can be explained in terms of increased capillary permeability or a low rate of return, or both. It has been known for a long time that young animals have relatively larger quantities of lymph than older ones (Emminghous, 1873). Holman (1937) found that lymph flow was doubled in growing dogs but that once they were mature

there was no significant change, even in very old dogs. Kruse (1970) found an increased permeability to Evans Blue in the neonatal calf. Many of the calves in this section also showed a significant loss of labelled material (IgG_1 or albumin) over the first 10 minutes after injection. This was why it was necessary to take several timed samples and extrapolate back to the plasma radioactivity at "t_o" (see Section II C 8a i).

There is thus evidence for increased capillary permeability and increased lymph flow. Further investigation of both factors is required before it will be possible to assess the significance of the increased capillary permeability in relation to the relatively larger extravascular compartment. Because of the lack of "Steady State" conditions, they cannot be determined by the method of Matthews (1957). A related phenomenon is the state of maturity of the "ground substance". A lot of work needs to be done on the physical forces operating in the extravascular compartment so that this possibility can only be conjecture at the moment.

In addition other factors will influence the situation. While not large enough to account for the total change in distribution, the decrease in plasma volume (ml/Kg) with age and the total body water as a fraction of body weight are both well recognised. Changes will also occur in the relative

amounts of different tissues, but this will be partly linked with the changes in body fluids with age.

The possibility that for IgG_1, the change is as a result of the lack of synthesis, particularly when the close correlation between catabolism and distribution is noted, can be discounted because of the similar change in albumin distribution, which is not correlated to catabolism but to plasma volume.

The albumin distribution in adult cattle and the calves in this study was determined by the equilibrium time method of Campbell et al and additional values determined by the Sterling method. The IgG distribution of Nansen and Nielsen (1966) and Nielsen and Nansen (1967) was determined by analysis of the plasma curve, which cannot be applied to the neonatal calf as it is completely dependent on "Steady State" conditions.

Other workers (Cohen, 1963, Wetterfors, 1965 and Plantin, 1966) have shown that where the Campbell, Sterling and Matthews methods for determining the distribution have been compared, the Sterling method tends to over-estimate the Extravascular compartment, although the results are still quite close. The other two methods show a good measure of agreement under a variety of disease conditions. The results from the repeat determinations (Table 5) indicate that there is a change with age. This, coupled with the agreement between the Campbell

and Sterling distributions makes it unlikely that the larger IgG_1 extravascular pool in the neonatal calf, can be accounted for in terms of the different methods of measurement.

The correlations between the Campbell and Sterling distributions, for IgG_1 and albumin indicate that the Sterling method can be used to determine the distribution of plasma proteins in the neonatal calf. It does not however allow for any increased catabolism over the period of distribution, as a result of diarrhoea or partial denaturation of the labelled preparation and must therefore be applied with caution. The positive correlations between the Campbell distribution and the four measures of catabolism of IgG_1, may reflect the lack of synthesis, which in older animals, will balance catabolism (under steady state conditions). Similar correlations were not found for albumin. In addition, as the catabolism of IgG_1 in the diarrhoeic calves was significantly increased (Section IV D) the distribution itself may also have been altered by the incidence of diarrhoea. For this reason, it was decided to use the E.V./I.V. ratio of 1.2/1 of the three non-diarrhoeic calves, for the subsequent colostral studies (see Section VI). A further four out of the remaining nine calves, had a similar distribution.

The significant negative correlation between the albumin

distribution (Campbell and Sterling) and the P.V. (ml/Kg) can be understood when it is realised that the plasma volume is being expressed as a fraction of the Total Body pool i.e. it is equivalent to I.V./I.V. + E.V., or 1/(1 + E.V./I.V.). Thus if the relative size of the extravascular pool increases, this fraction must decrease i.e. the P.V. (ml/Kg) will decrease. The same relationship does not exist for IgG_1, presumably because of the close relationship between the distribution of IgG_1 and catabolism.

The very close correlation found between the F.C.R. and the Total Body C.R. in Groups 1 and 2 and to a lesser extent in Group 3 has a bearing on the variability or otherwise of the relative size of the extravascular compartment. Thus Andersen (1964) explained the poor relationship between these two parameters in human metabolic studies, in terms of the considerable variation that exists in the relative size of the extravascular pool. In the neonatal calf, the amount of variation must therefore be small, particularly for IgG_1. This will be important in colostral studies where the previously determined distribution will be used to calculate the absorption efficiency. Also it justifies the use of the Total Body C.R. and the Total Body $T\frac{1}{2}$ as measures of catabolism as well as the F.R.C. and the Plasma $T\frac{1}{2}$, in the neonatal calf.

Of the four measures of catabolism, the plasma $T\frac{1}{2}$ would appear to be the least satisfactory (see Section IV D). Other workers have found considerable variations when so called plasma $T\frac{1}{2}$'s are compared and they thus prefer not to use this parameter at all. In this section, it has been referred to as the apparent plasma $T\frac{1}{2}$. It will have been influenced by changes in plasma volume associated with growth and / or diarrhoea.

To take full account of the changes in plasma volume over the 14 day experimental period, it would have been necessary to measure the plasma volume every 24 to 48 hours. As the majority of calves in all these groups had diarrhoea at some point during the experimental period, plasma volume fluctuations as part of the general disturbance of body fluids, will have occurred. These would not have been taken into account if the plasma volume had been measured twice, at the beginning and end of the 14 day experimental period and a daily correlation factor then calculated. Thus in those calves where the plasma volume was determined at the end of the experiment no attempt was made to apply a correction to the daily plasma radio activity levels i.e. to Qp.

The close agreement for IgG_1 and Albumin between the Sterling and Campbell distributions and for IgG_1, the highly

significant correlations between the four measures of catabolism, two of which depend on Qp would appear to justify the ommission of the plasma volume changes in these studies.

The plasma volumes measured with isotopically labelled IgG_1 and albumin (Tables 3 and 7) were considerably lower than the plasma volumes of approximately 90ml/Kg, reported by McEwan, Fisher and Selman (1968). These workers used Evans Blue and calculated the plasma volume from a single 10 minute sample, without making any allowance for losses from the circulation during this period. Such losses have been allowed for in the isotopic studies.

As already indicated in the Introduction, it is not possible to apply all the standard methods for evaluating the metabolism of plasma proteins to the neonatal calf. In the healthy adult animal, the serum concentration of any protein is merely a reflection of the dynamic equilibrium that exists between catabolism and synthesis i.e. the turnover of the protein. Thus if the animal under study is in a steady state with respect to the protein under study, the magnitude of the turnover rate can be determined from a measure of either synthesis or catabolism. Usually catabolism, the amount or fraction of the protein broken down per unit time, is measured.

In the neonatal calf we have several problems, namely the lack of synthesis of IgG_1, the change in body weight - which, in these studies, was quite variable and the possible influence of diarrhoea. Any one of these factors means that steady state conditions do not exist. Taken together, the situation is even further removed from that envisaged by Matthews, Reeve and others who have analysed plasma protein metabolism in very great detail, under steady state conditions.

Thus although the model on which the Campbell method is based oversimplifies the situation and is thus discarded by Takeda and Reeve (1963) as having no physiological reality, for the purposes of these studies, it had a number of advantages. Provided the change in the pool sizes, as a result of one or more of the factors mentioned above is not too rapid or large, it will determine the distribution. While the accuracy of this determination may not be as high as that obtained by Matthews, Reeve and others it has the supreme advantage that it can be used, whereas the other methods are completely unacceptable.

The Sterling method is based on the assumption that catabolism takes place equally in both intra and extravascular pools, which is known not to be the case. However, for both

plasma proteins investigated it gives very similar results, agreement being closer for albumin than for IgG_1. This is possibly due to the influence of diarrhoea in the first few days of the experiment, which would be allowed for in the Matthews calculations, but would appear to give a larger extravascular pool by the Sterling method.

Over and above these considerations, it must be borne in mind that the purpose of these studies is to be able to quantitate the efficiency of absorption of colostral IgG_1. Thus although Matthews will not allow for slowly exchanging parts of the extravascular pool, what we want to know is the distribution of the largest part of the IgG_1 absorbed, before catabolism and indeed variations in catabolism start to significantly alter the levels of IgG_1. Thus any slowly exchanging part of the E.V. pool is not as important and can probably be ignored.

SUMMARY.

In this Section all the information required for a study of the efficiency of absorption of colostral Fast IgG was obtained. The distribution (E.V./I.V.) of Fast IgG (IgG_1) in the calf was found to be 1.2/1 . For albumin, the ratio was 1.9/1 . Equilibrium was complete by 48 hours. The older the calf, the lower the ratio for IgG_1 . A close relationship was found between the distribution of IgG_1 and the level of catabolism. However, the increase in the ratio associated with hypercatabolism of IgG_1 in the diarrhoeic calves was small, and not significant. The relationship between the Catabolic Rates, for both proteins studied, provided additional evidence that the distribution of IgG_1 in the neonatal calf, is not subject to much variation.

SECTION V

THE METABOLISM OF BOVINE IgM

INTRODUCTION

In Section I the gradual identification of a separate 19s (IgM) class of antibodies was traced. Although the early work of Kabat and Pedersen had established that certain antibodies, in some species were macroglobulins, it was only 20 years later with the work of Franklin, Kunkel and others that this 19s fraction was recognised as a normal constituent of γ globulin. In two reviews, Kunkel (1960) and Kunkel, Fudenberg and Ovary (1960), Kunkel differentiated between γ_1 and α_2 macroglobulins, of which the γ_1 group were closely related to certain pathological Waldenström macroglobulins. He concluded that 19s γ_1 macroglobulins constituted a distinct class of antibody and classified them on the basis of their high molecular weight, electrophoretic and antigenic properties, carbohydrate content etc. Antibodies against whole bacteria and red cells were identified in this class. The development of improved separation techniques, particularly molecular sieve chromatography made the preparation of 19s fractions much easier (see also Section III - Introduction).

The preparation of IgM from serum usually requires the combination of several methods. Thus Muller-Eberhard, Kunkel and Franklin (1956) used a combination of zone electrophoresis and repeated ultra-centrifugation. Similarly Kunkel (1960)

described the use of density gradient ultracentrifugation. Bauer, Mathies and Stavitsky (1963) combined the two methods of Muller-Eberhard et al (1956) with ion-exchange chromatography. Caputo, and Appella (1960) isolated a human macroglobulin by euglobulin precipitation, followed by dialysis against saturated ammonium sulphate. A similar method was used by Truax, Bray and Koenig (1962). Olesen (1963) obtained a partially denatured IgM using a variety of techniques including initial euglobulin and ammonium sulphate precipitations, as well as cold agglutinin adsorption by red cells. Wilkinson, Davidson and Sommaripa (1966) used a euglobulin precipitate for their metabolic study of autologous ^{131}I - labelled macroglobulin.

Kochwa, Rosenfield, Tallal and Wasserman (1961) separated iso-agglutinins on DEAE cellulose into 2 groups, the second fraction eluted with 1M NaCl, containing 19s macroglobulins. For pathological sera they combined it with a euglobulin precipitation. A similar method was used by Gabuzda (1962). Murphy et al (1965) identified γM in peak III of their DEAE Sephadex chromatogram. Robbins, Kenny and Suter (1965) isolated rabbit γM antibodies using DEAE cellulose.

Apart from these three methods, used alone or virtually so, many workers have used molecular sieve chromatography combined with other techniques. Flodin and Killander (1962)

and Fireman, Vannier and Goodman (1964) using Sephadex G-200, obtained a first peak containing 2 main components, in addition to lipoprotein. Fahey and Solomon (1963) found 2 antigenically different types of γ_1 macroglobulin, starting with a euglobulin precipitate, followed by ammonium sulphate precipitation or zone electrophoresis and finally chromatography with DEAE cellulose or Sephadex G-200. Killander (1963) and Barth, Wochner, Waldmann and Fahey (1964) combined gel filtration with block electrophoresis. Murphy et al (1964b) and (1965) identified Bovine IgM in the first peak from Sephadex G-200 and in fractions obtained by zone electrophoresis.

Ahlinder, Birke, Norberg, Olhagen and Plantin (1965) after separating IgM on G-200, used albumin as a protective colloid, before labelling with ^{125}I and removal of unbound iodide. Chaplin, Cohen and Press (1965), after initial ultracentrifugation to remove the lipo-proteins, carried out a G-200 separation. After labelling with ^{125}I and removal of unbound iodide, final purification was carried out by block electrophoresis. Birke, Norberg, Olhogen and Plantin (1966), for their study of the metabolism of human IgM preceeded a G-200 separation by zone electrophoresis. Deutsch (1967) purified his euglobulin precipitate by gel filtration. He found that 65 to 85 % was 19s and the rest polymers. Seligmann

and Mihaesco (1967) used multistage processes involving most of the techniques already mentioned, to prepare IgM for structural studies. McDonagh and Inman (1970) similarly prepared rabbit IgM. Bradley (1969) used the method of Barth et al (1964). Jensen (1969b) separated lipoprotein depleted human serum on Sephadex G-200 and recycled the first peak on Sepharose 4B. The remaining α_2 macroglobulin was removed on DEAE cellulose. Jonas (1969) separated Ovine IgM on Sephadex G-200. Using sucrose density gradient ultra-centrifugation, followed by gel filtration and immunogel-filtration, Solomon (1969) distinguished a 7s prehein with γ M antigenic determinants. Johnston and Miller (1970) sepatated pathological macroglobulins on G-200, using a euglobulin fraction. Finally, recycling on Sepharose 4B yielded a preparation, 98% pure.

Most of the early work on the catabolism of γ M was carried out using pathological macroglobulins, in human patients suffering from Waldenstroms macroglobulinaemia. The results therefore must be treated with caution. Truaz et al (1962) showed that the same preparation gave a plasma half life of 14 days in a patient, compared with 6 - 7 days in a normal subject. Thus the results of Drivsholm (1961), Gabuzda (1962), Olesen (1963) and Wilkinson et al (1966) must be treated with

care. The general conclusion however, that this plasma protein is largely confined to the vascular compartment and has a shorter plasma half life, associated with a high fractional catabolic rate (compared to IgG), was supported by the work of Cohen and Freeman (1960) and Barth et al (1964). Barth et al found, that approximately 80% (65 to 100) of the protein was intravascular, plasma half lives of 3.8 to 6.5 days and F.R.C.'s of 25 to 14 %/day. The same workers found that IgM was rapidly catabolised in patients with protein-losing gastroenteropathy.

Stiehm, Vaerman and Fundenberg (1966) found a mean plasma $T\frac{1}{2}$ of 9.6 days (7 - 11) for IgM in agammaglobulinaemic patients treated with whole serum. Subsequent studies, Birke, Norberg, Olhagen and Plantin (1967), Strober, Wochner, Carbone and Waldmann (1967), Jensen (1968) and (1969a and b), Olesen and Hippe (1968) and (1969), Bradley (1969) and Jensen, Goltermann, Jarnum, Weeke and Westergaard (1970) have produced further evidence that IgM is largely confined to the vascular compartment in man. Thus Bradley (1969) obtained distributions in the range 65 to 70% intravascular and Strober et al (1967) of 76 ± 12 %. Half lives and F.R.C.'s have varied, probably depending on what proportion of the preparation was partly denatured. On the one hand, Bradley obtained plasma $T\frac{1}{2}$'s

of 3.7 to 6.5 days and on the other, Jensen (1969a) of approximately 10 days. F.R.C.'s show an even greater variation, the range of 8 to 20 % determined by Olesen and Hippe (1969) representing both extremes.

Rheumatoid Factor in man is well recognised as being predominantly an abnormal IgM component associated with Rheumatoid Arthritis (Edelman, Kunkel and Franklin, 1958, Svartz, 1960, Killander and Philipson, 1964, Squire, 1966 and Bradley, 1969). Bradley, in his metabolic studies obtained very similar results for IgM and Rheumatoid Factor.

Similar distributions have been found for Fibrinogen (McFarlane, 1963, McFarlane, Todd and Cromwell, 1964, Atencio and Reeve, 1965 and Takeda, 1966 and 1970). Norberg, Birke, Hedfors and Plantin (1970) found that 92 to 94 % of the α_2 macroglobulin is intravascular.

There was no information available concerning the catabolism of Bovine IgM, On the basis of human work, it seemed reasonable to expect Bovine IgM to be largely intravascular in distribution. From the review of the different methods of preparation, it can be seen that most workers have used molecular sieve chromatography with or without additional proceedures to isolate the immunoglobulin. It was therefore decided to separate Bovine IgM in a similar manner using Sephadex G-200.

MATERIALS AND METHODS

All the calves used in these experiments, with the exception of C 9 (an Ayrshire heifer calf) were Ayrshire bull calves.

To ensure that the results obtained with the isotopically labelled preparation were reproducible, it was necessary to use several fractions, prepared separately, and then compare the results. To reduce the α_2M Macroglobulin level to a minimum without having to employ an additional separation, colostral whey was used as starting material. Lipoprotein present would tend to precipitate out, during preparation.

As the possibility of denaturation of the IgM itself would have to be considered, four criteria were chosen that would enable comparisons to be made between preparations, based on those recommended by Freeman, (1970).

These were:

Plasma Volume (P.V.) Deviation (%) i.e. the apparent percentage increase or decrease in plasma volume determined with the isotopically labelled IgM, compared to that measured with similarly labelled IgG, or the dye Evans Blue.

Plasma activity at 50 hours (%), to indicate the rate at which the preparation disappeared from the circulation.

Excreted activity (%) for Day 1 and Day 2 of the experiment to demonstrate any increase in the first 24 hours.

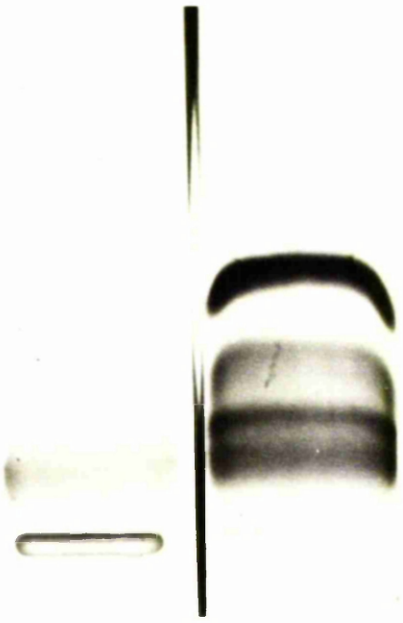
Fluctuations in the daily Fractional Catabolic Rate (see Section II C 8b iii).

For the calculation of the number of atoms of Iodine/molecule of labelled protein, see Dargie, 1969.

Figure 15

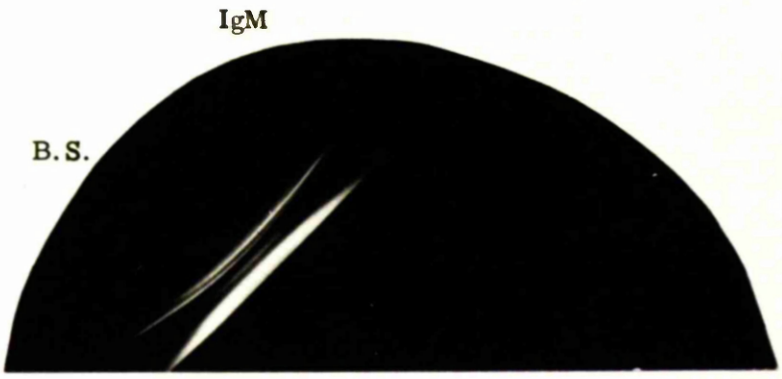
Examination of Bovine IgM prior to Isotopic Labelling

1. Electrophoresis



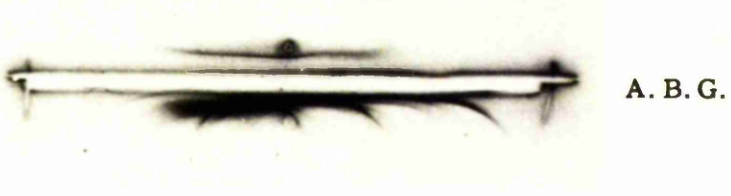
IgM B.S.

2. Double Diffusion



A. B. G.

3. Immunoelectrophoresis



B.S. - Bovine Serum

A. B. G. - Anti-bovine-globulin (with some anti-albumin activity)

A single broad precipitin line was obtained on Double Diffusion (2).

A. Initial IgM Study

1. Source of Calves

The calves were market calves, purchased through a dealer. They were up to one week old.

2. Source of IgM

50 ml of colostral whey was separated on Sephadex G-200 (see Section III A and B). The bed volume was 90 x 4.5 cm and the flow rate approximately 40 ml/hour. Two separations were carried out simultaneously. The break through peaks were concentrated with Sephadex G-25.

3. Examination of the IgM Preparation No.(i)

The IgM preparation was subjected to electrophoresis, double diffusion, immunoelectrophoresis and tested for anti-trypsin activity (see Section IV) as previously carried out for IgG_1 (see Fig. 15).

4. Labelling

(see Section II C 1)

The number of atoms of iodine incorporated was approximately 1.5/molecule of IgM.

5. Rechromatography of Labelled IgM

This was carried out as described in Section IV for IgG_1. Fig. 16 shows the chromatogram with the position of the labelled material superimposed.

Figure 16

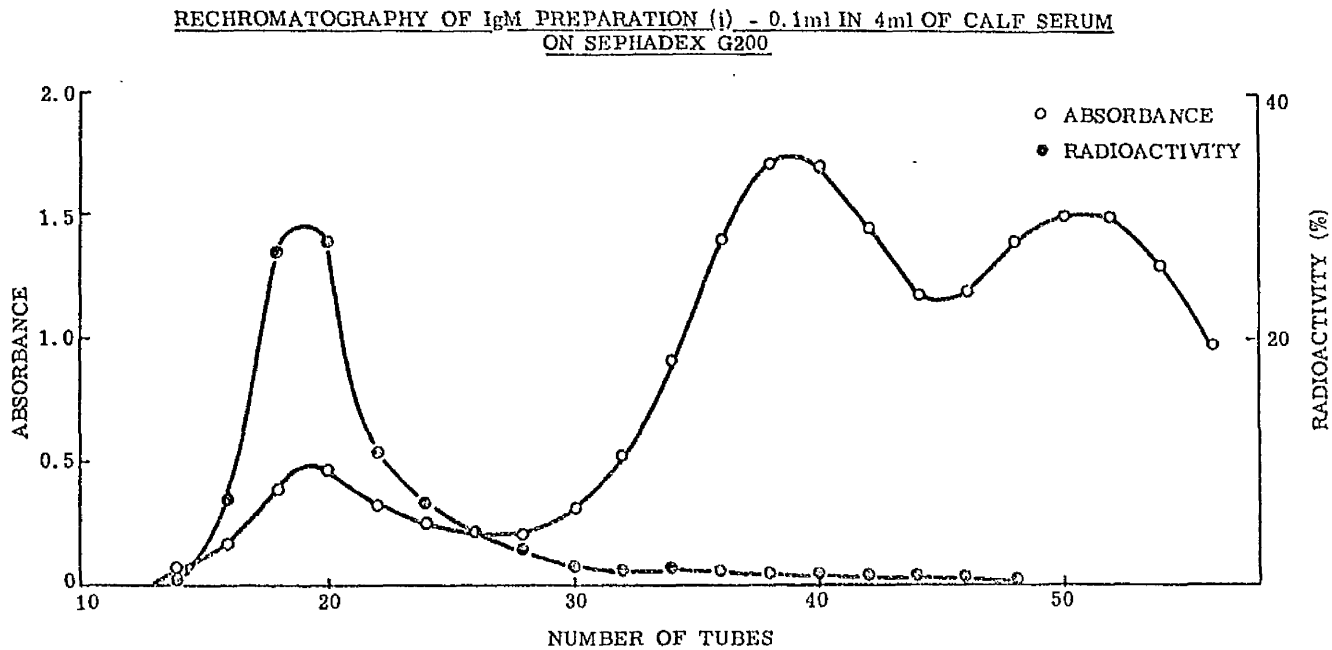

RECHROMATOGRAPHY OF IgM PREPARATION (i) - 0.1ml IN 4ml OF CALF SERUM ON SEPHADEX G200
ABSORBANCE
RADIOACTIVITY
ABSORBANCE
RADIOACTIVITY (%)
NUMBER OF TUBES
2.0
1.5
1.0
0.5
0
40
20
10
20
30
40
50

Table 9

The Distribution and Catabolism

of Labelled IgM preparation (1) in 4 Calves

Calf	I.V./E.V. (Sterling)	I.V./.E.V. (Campbell)	Apparent Plasma $T_{\frac{1}{2}}$ (days)	Total Body $T_{\frac{1}{2}}$ (days)
E	1/1.6	1/0.85	2.1	2.2
F	1/1.9	1/0.97	3.5	2.4
G	1/0.7	1/0.48	2.7	2.5
H	1/1.6	1/0.88	3.2	2.8
Mean	1/1.5	1/0.80	2.9	2.5
S.D.±	/0.51	/0.21	0.59	0.25
S.E.±	/0.25	/0.10	0.30	0.12

Figure 17

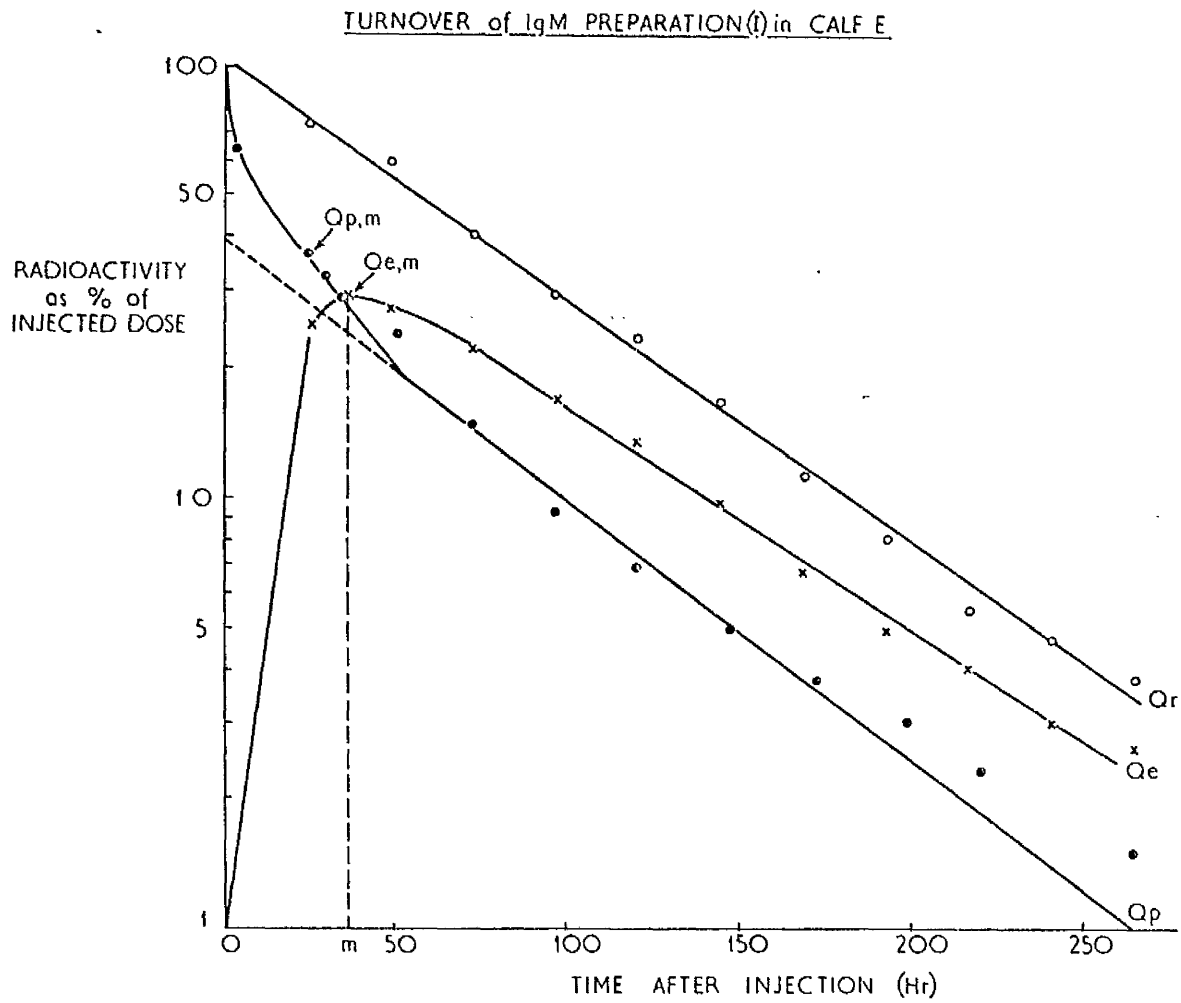
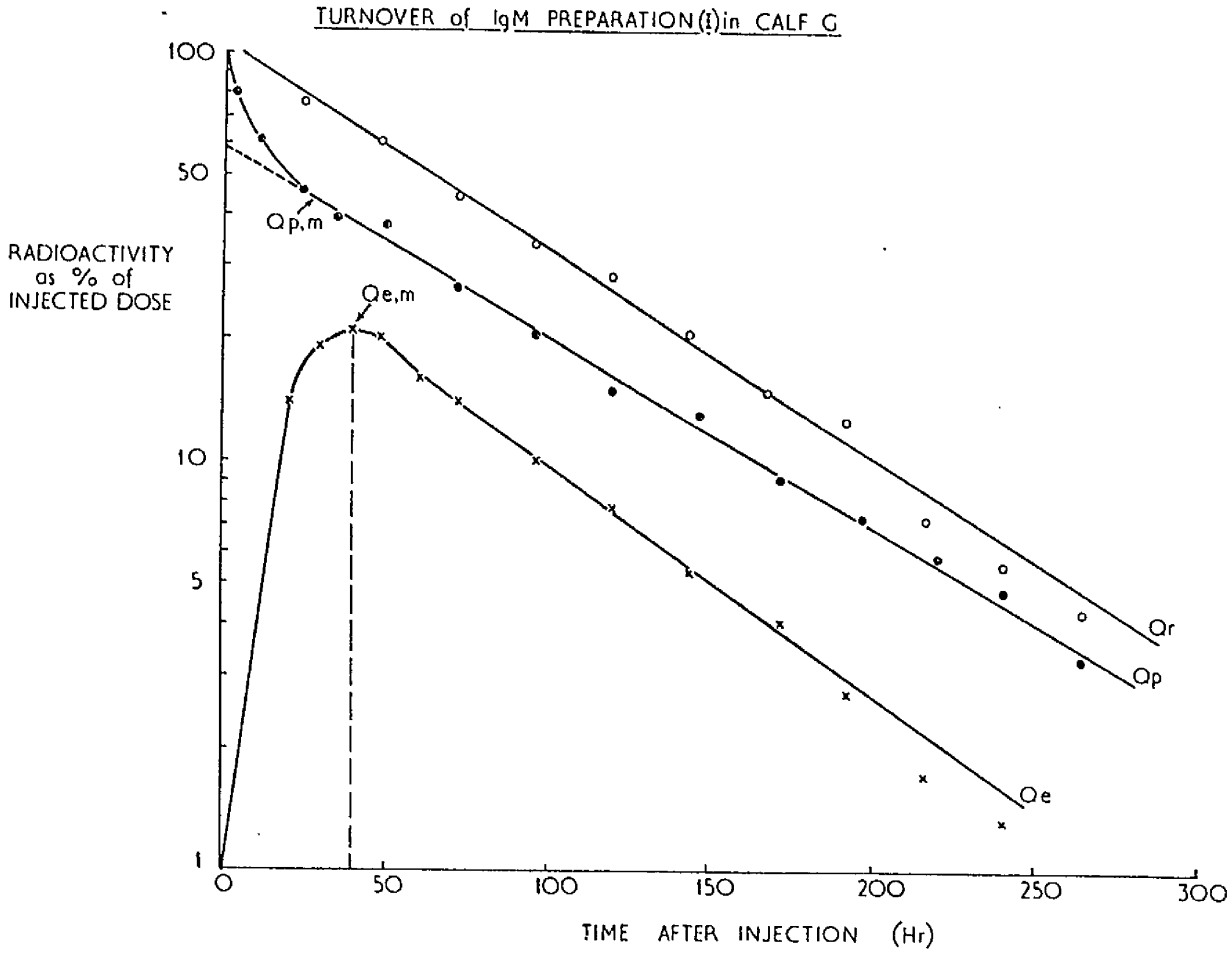

TURNOVER of IgM PREPARATION(I) in CALF E
100
50
10
5
1
RADIOACTIVITY
as % of
INJECTED DOSE
Qp,m
Qe,m
Qr
Qe
Qp
0
m
50
100
150
200
250
TIME AFTER INJECTION (Hr)

Figure 18


TURNOVER of IgM PREPARATION (I) in CALF G
RADIOACTIVITY as % of INJECTED DOSE
100
50
10
5
1
0
50
100
150
200
250
300
TIME AFTER INJECTION (Hr)
Qp,m
Qe,m
Qr
Qp
Qe

6. Injection

Each calf received approximately 300μc of ^{125}I - labelled IgM.

7. For all other experimental details, see Materials and Methods, Section IV.

Results

(see Tables 9 and 10, and Figures 17, 18 and 19)

The apparent distributions (I.V./E.V.) as determined by both Campbell and Sterling methods, are shown in Table 9, along with the Apparent Plasma $T\frac{1}{2}$'s. A marked discrepancy was found in the apparent distribution, calculated by the two methods. Thus the Campbell distribution of 1/0.8 ± 0.21 indicated a relatively larger intravascular pool. The Sterling distribution of 1/1.5 did not show this. Plasma and Total Body $T\frac{1}{2}$'s of 2.9 ± 0.59 days and 2.5 ± 0.25 days respectively, were of a similar order. As the calves were diarrhoeic, these are likely to represent about half control values.

There was an apparent increase in plasma volume of 10.3 ± 4.3 (%), and only a quarter of the labelled material (± 6.0%) was still present in the circulation at 50 hours. The level of excreted activity on Day 1 was significantly higher than on Day 2 ($p=<0.001$). Although the daily

FIGURE 19

THE DAILY VARIATION OF THE FRACTIONAL CATABOLIC RATE (F.C.R.) OF IgM PREPARATION (i) IN 4 CALVES

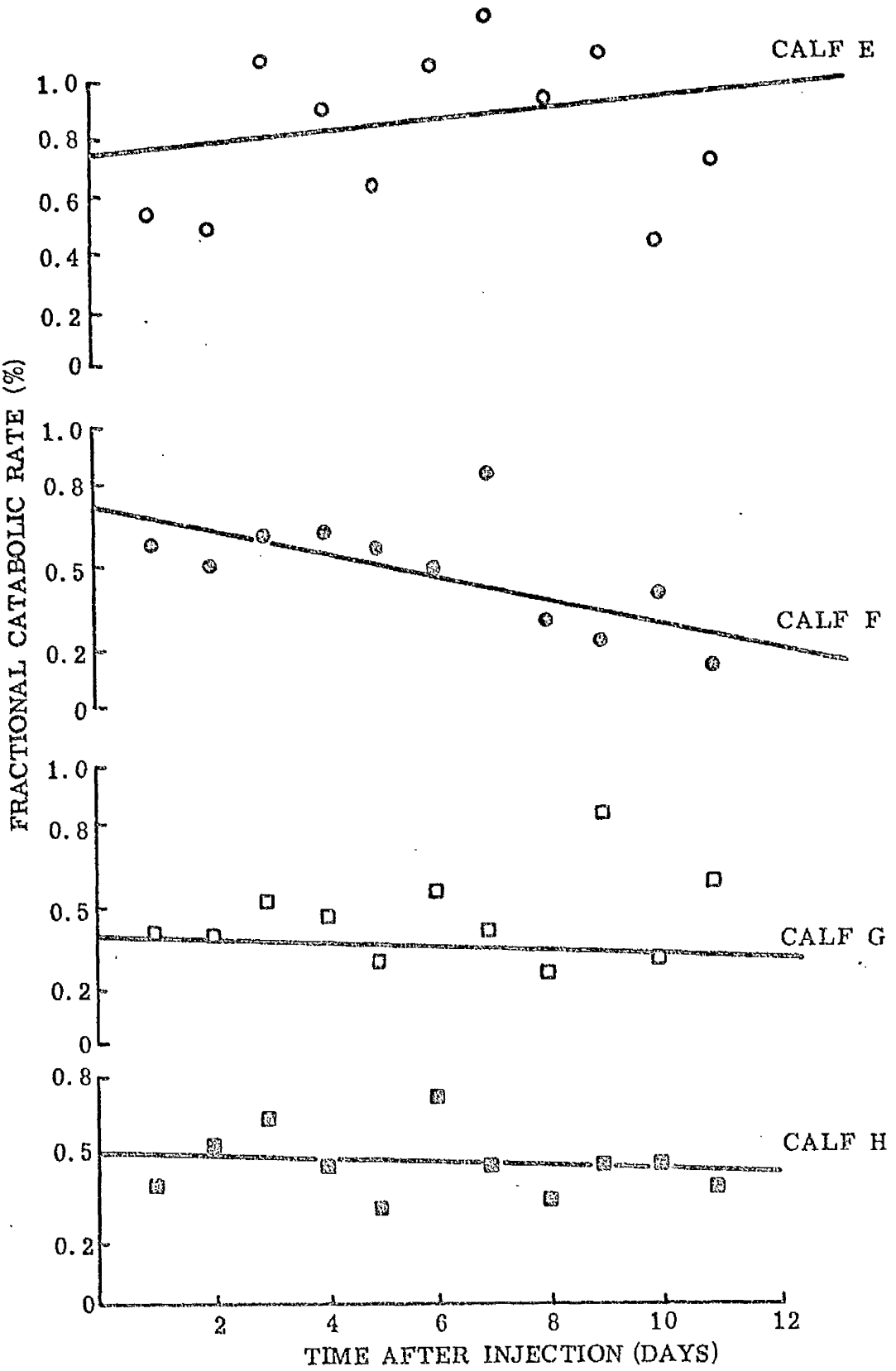


Table 10

The Denaturation Criteria applied to IgM preparation (i)

Calf	P.V. Deviation (%)	Plasma Activity at 50 hours (%)	Excreted Activity (%)	
			Day 1	Day 2
E	13.5	21	27	13
F	14.2	22	33	14
G	8.1	34	24	16
H	5.3	24	29	14
Mean	10.3	25	28	14
S.D.±	4.3	6.0	3.8	1.3
S.E.±	2.1	3.0	1.9	0.6

fractional catabolic rate (F.R.C.) varied considerably (see Fig 19), it was relatively constant in calves G and H. It increased in Calf F, but after an initial steady phase, it started to fall 4 days before the calf died.

The Plasma Protein and P.V.C. figures for these calves are in Table 10.

Conclusions

The apparent increase in plasma volume and the raised level of excreted activity in the first 24 hours would seem to indicate a degree of gross denaturation. The F.C.R.'s decreased in only one calf, and then prior to death, indicating that the remainder of the preparation not immediately excreted in the urine was fairly metabolically homogenous. The possibility that it was uniformly denatured to an extent, cannot be excluded. The discrepancy between the Campbell and Sterling distributions is explicable in terms of the high initial catabolism, which is allowed for when calculating the Campbell distribution.

Figure 20

RECHROMATOGRAPHY OF IgM PREPARATION (ii) - 0.1ml IN 4ml OF CALF SERUM ON SEPHADEX G200

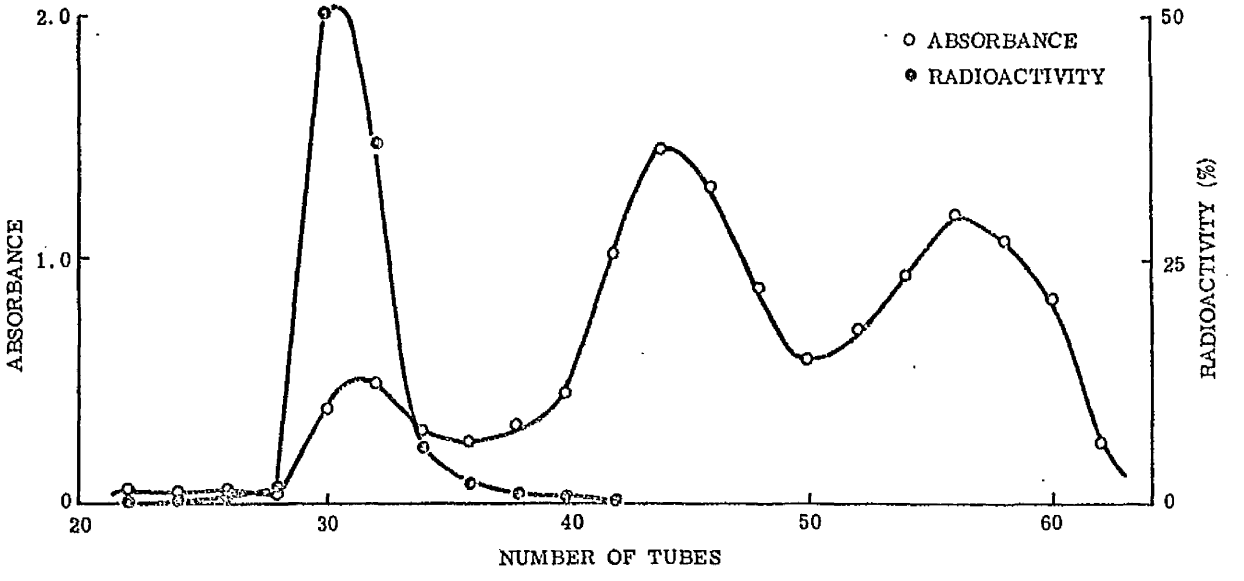


Table 11

The Distribution and Catabolism of Labelled IgM

Preparation (ii) in 4 calves

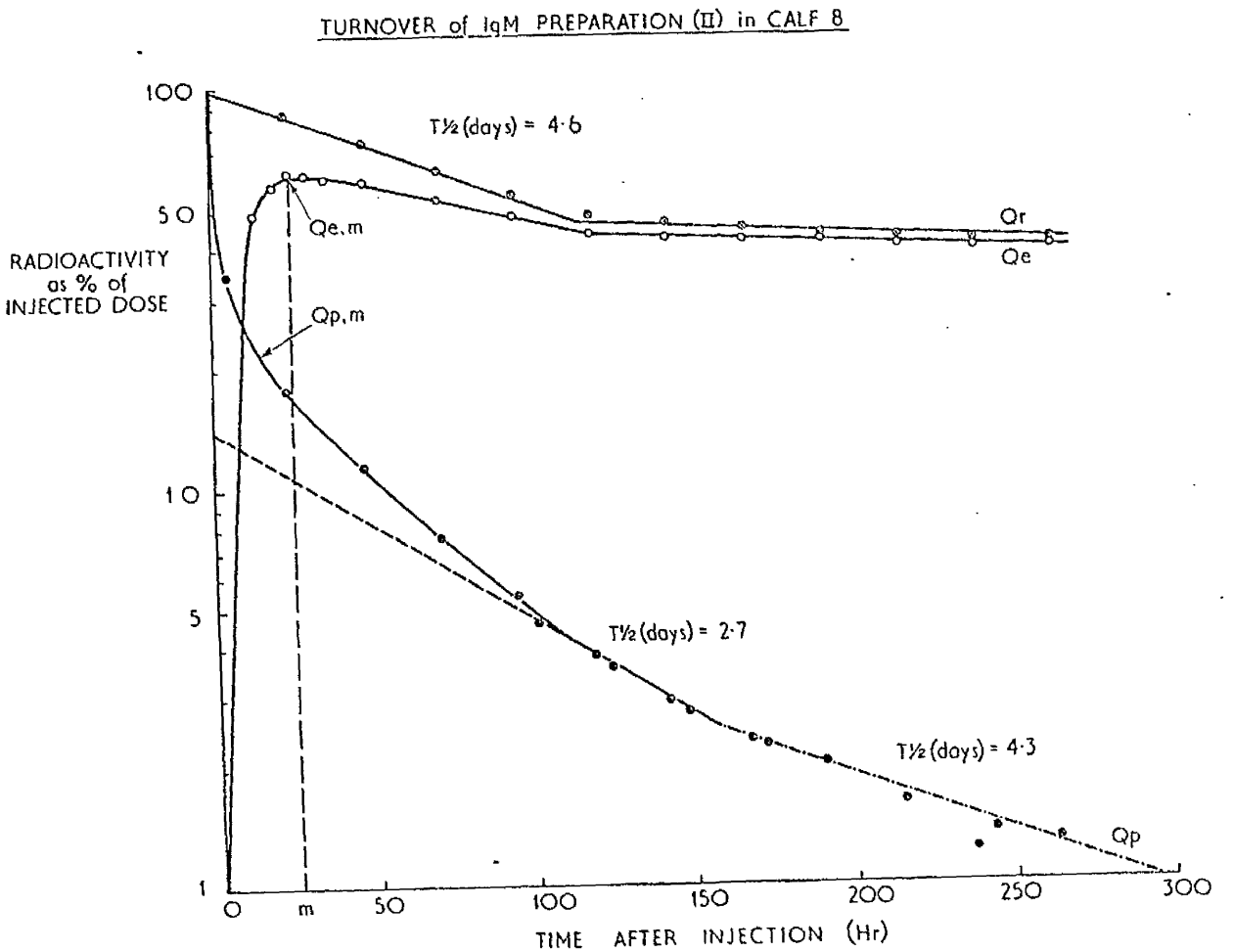
Calf No.	I.V./E.V. Sterling Ratio	I.V./E.V. Campbell Ratio	Plasma $T_{\frac{1}{2}}$'s (days) Initial	Final	Initial Total Body $T_{\frac{1}{2}}$ (days)
8	1/6.1	1/2.5	2.7	4.3	4.6
9	1/4.3	1/2.6	1.8	8.8	5.8
10	1/6.7	1/5.3	2.9	7.9	6.8
11	1/3.5	1/5.9	2.9	8.3	6.2
Mean	1/5.2	1/4.1	2.6	7.3	5.9
S.D.±	/1.5	/1.8	0.53	2.0	0.93
S.E.±	/0.7	/0.9	0.26	1.0	0.46

Table 12

The Denaturation Criteria applied to Labelled IgM preparation (ii)

Calf No.	P.V. Deviation (%)	Plasma Activity at 30 hours (%)	Excreted Activity (%)	
			Day 1	Day 2
8	101	10.5	13	14
9	145	18.0	12	15
10	167	11.3	7	10
11	188	11.5	9	9
Mean	150	12.8	10.3	12.0
S.D.±	37	3.5	2.8	2.9
S.E.±	19	1.7	1.4	1.5

Figure 21


TURNOVER of IgM PREPARATION (II) in CALF 8
100
50
10
5
1
RADIOACTIVITY as % of INJECTED DOSE
T½ (days) = 4·6
Qr
Qe
Qe,m
Qp,m
T½ (days) = 2·7
T½ (days) = 4·3
Qp
0
m
50
100
150
200
250
300
TIME AFTER INJECTION (Hr)

B. First IgM Repeat Study

1. Source of Calves

The calves were purchased from Mr H. Guthrie, Moss Side Farm, Kilmarnock. They were 2 to 5 days old.

2. Source of IgM — preparation no. (ii)

The method of preparation was the same as for the initial study (see above) except that it was necessary to rechromatograph the preparation to remove contaminating IgG_1. It was stored at $-20^{\circ}C$.

3. Injection

Each calf received approximately 1mc of ^{125}I - labelled IgM.

4. For all other proceedures, see Section V A above.

Results

(compared to the initial study - Tables 11 and 12 and Figures 21 and 22).

This time, the apparent plasma volumes were very considerably increased i.e. 150 ± 37 %, and the plasma activity at 50 hours correspondingly reduced to 12.8 ± 3.5 %. There was however little difference between the Day 1 and Day 2 excreted activity. The F.R.C.'s showed a greater variation (see Fig 22) than in the initial study. When the effects of the initial

Figure 22

THE DAILY VARIATION OF THE FRACTIONAL CATABOLIC RATE (F.C.R.) OF IgM PREPARATION (ii) IN 4 CALVES

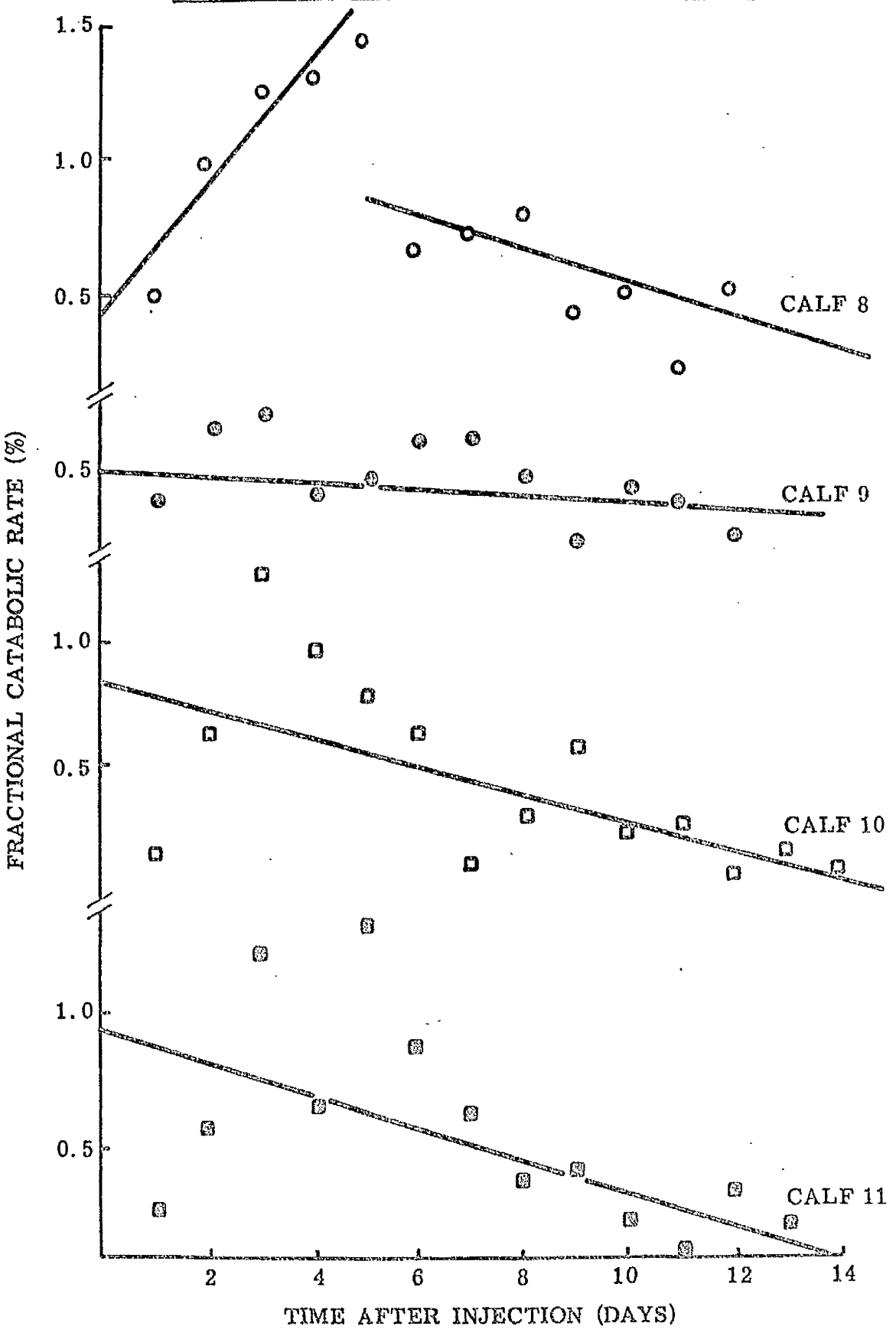


Table 13

The Plasma Protein Results (gm/ml) and mean P.C.V. for Calves in Tables 11 & 12

Calf No.	Mean P.C.V.	Day 0 Total Protein	Day 0 A/G Ratio	Day 7 Total Protein	Day 7 A/G Ratio	Day 14 Total Protein	Day 14 A/G Ratio
8	43	4.0	0.87	3.5	0.74	3.9	0.63
9	36	4.4	0.94	4.0	0.73	4.5	0.69
10	31	4.8	0.70	4.4	0.75	4.4	0.83
11	30	4.4	0.80	4.1	0.70	4.6	0.73
Mean	35	4.4	0.83	4.0	0.73	4.4	0.72
S.D.±	5.9	0.33	0.10	0.37	0.02	0.31	0.08
S.E.±	3.0	0.16	0.05	0.19	0.01	0.16	0.04

rises in calves 8, 10 and 11 (possibly associated with diarrhoea) were removed, all four calves showed a falling F.C.R. , although in calf 9 it was only slight.

The apparent distribution by both methods was very different from that found in the initial study. This time there was an apparently very large extravascular pool i.e. 1/5.2 (Sterling) and 1/4.1 (Campbell). The plasma disappearance curve (Fig. 21) consisted of two components with half lives of 2.6 ± 0.53 days and 7.3 ± 2.0 days. The plasma protein and P.V.C. figures for these calves are in Table 13.

Conclusions

The plasma volume deviation and the low plasma activity at 50 hours indicated that a large proportion of the labelled IgM was grossly denatured. As the F.C.R.'s did not become constant after the removal of the grossly denatured material, the remaining IgM was probably also damaged to an extent. The final plasma $T\frac{1}{2}$ of 7.3 ± 2.0 days may however give some indication of the likely disappearance of undenatured IgM from the circulation. In view of the denaturation, it is hardly surprising that the apparent distribution of the preparation was very different from the initial study. It can be seen from Fig. 21 that even at the end of the experiment, about half of the isotopic label is still retained extra-vascularly

(30 to 60 % of the injected dose).

The only differences between the two preparations were firstly that the IgM used for the repeat experiment had been rechromatographed on G-200 and secondly, it had been stored at -20°C. It may therefore be concluded that one or both of these proceedures contributed to the increased denaturation of the IgM used for the first repeat study. Thus, while the results from the initial experiment had been moderately encouraging, the repeat experiment indicated that a considerable degree of denaturation had occurred. Although it might be possible to make valid deductions from an experiment in which only a proportion of the labolled material were denatured, or in which the general level of denaturation were slight, neither preparations would be suitable for a study of the efficiency of absorption of colostral IgM.

While no information was available for Bovine IgM, work with human IgM had indicated that the majority of it was retained intravascularly. Thus a preparation suitable for colostral efficiency studies would not disappear rapidly from the circulation i.e. the plasma activity would be at least 50% of the initial activity or even higher. Also, the Plasma $T\frac{1}{2}$ might be expected to be of the order of 10 days. An indication of the suitability of a preparation could be

obtained from the apparent plasma volume, plasma activity at 50 hours and subsequent plasma $T\frac{1}{2}$.

Both the initial and first repeat IgM experiments had been carried out with IgM labelled with 1.5 atoms of iodine per molecule. Freeman (1966) stated that for human IgM, the level of iodination should be kept to 0.5 atoms per molecule. Although other factors had already been implicated as possible causes of gross denaturation there was still a degree of denaturation in the initial experiment to be accounted for. Apart from the method of preparation of the IgM itself, the level of iodination (1.5 atoms /molecule) would appear to be the next most likely cause.

Table 14

The Plasma Disappearance of Labelled IgM in 2 Calves, varying the level of Iodination

	Calf No.	P.V. (ml/Kg)	Plasma Activity at 50 hrs (%)	Plasma T½'s Initial	Final
1.5 atoms I/molecule	9	63.5	34.5	5.0	19.6
	13	64.9	28.0	5.9	18.0
0.8 atoms I/molecule	9	45.5	22.5	8.4	17.6
	13	61.7	27.2	6.4	21.2
0.4 atoms I/molecule	9	38.2	23.5	9.1	
	13	52.7	30.2	7.1	

C. Second IgM repeat study

(varying the level of iodination)

1. Source of Calves

The two calves used were 22 weeks old (calf 9) and 19 weeks old (calf 13) respectively at the start of the experiment (see Table 14). They had been retained after the IgG_1 experiments - see Section IV.

2. Source of IgM

The method of preparation used and the proc edure followed were the same as employed in the initial experiment A.

3. Iodination

To enable a valid comparison to be made in these older calves, the first preparation used was labelled with the original level of 1.5 atoms/molecule (each calf receiving about 600µc of ^{125}I labelled IgM). Three weeks later, the same calves were injected with 100µc of ^{131}I labelled IgM (0.8 atoms per molecule) and 100µc of ^{125}I labelled IgM (0.4 atoms per molecule).

4. For other proceedures, see Section V A .

Results

(see Tables 14 and 15)

Although there was an inevitable delay between the two

Table 15

Additional Information about the 2 Calves in the 2nd IgM Repeat Study

	Calf No.	Mean P.C.V.	Total Protein (gm/100ml)	A/G Ratio	Age (weeks)
1.5 atoms I/molecule	9	28	6.3	0.58	22
	13	30	7.9	0.39	19
0.8/0.4 atoms I/molecule	9	27	6.3	0.58	26
	13	28	7.5	0.39	23

parts of the experiment, a direct comparison between the results was still possible. Both calves showed apparent reductions in the plasma volume using the two preparations with the lower number of atoms of Iodine per molecule.

When expressed as % increases over the lowest values they represented increases of 66% (1.5 atoms/molecule) and 19% (0.8 atoms/molecule) for C 9, and similarly 23% and 17% for C 13. Comparisons on the basis of the Plasma Activity at 50 hours and the initial plasma half-life are difficult because of the differences between the two calves. All the 50 hour plasma activities are close to the mean value obtained in the initial IgM study of 25% ± 6%. The initial plasma $T\frac{1}{2}$'s show a tendency to be longer, at the lower iodination levels. This is most marked in C 9 but when taken in conjunction with the higher 50 hour plasma activity, may be the result of other factors. All the initial plasma $T\frac{1}{2}$'s are longer than those found in the earlier IgM studies (2.9 ± 0.59 days and 2.6 ± 0.53 days, respectively) and are closer to the final plasma $T\frac{1}{2}$ in the first repeat study of 7.3 ± 2 days.

Thus only the plasma volume results give a clear indication of the influence of the higher levels of iodination. The result would seem to be that in these particular studies, the IgM molecules thus denatured are very rapidly removed from

the circulation. The final plasma $T_{\frac{1}{2}}$'s are longer than might have been expected. They may, at least in part, represent a 19s form of IgG, as described by Hammer, Kickhofen and Hemming (1968). In view of the results in this investigation of the influence of different levels of iodination, it was decided that, in future, Bovine IgM preparations would be labelled with 0.5 or less atoms of Iodine per molecule.

D. Third IgM Repeat Study

1. Source of Calves

The calves were purchased from Mr H. Guthrie, Moss Side Farm, Kilmarnock. They were one week old at the start of the experiment.

2. Source of isotopically labelled IgM - no (iii)

The starting material was colostral whey (1 litre). An initial fractionation was carried out on DEAE A-50 Sephadex. After elution with 0.15M phosphate buffer, the IgM enriched fraction was eluted with 0.5M NaCl (i.e. the step wise instead of gradient elution). The middle of this peak was concentrated in an ultrafiltration cell (Millipore U.K., London) and then labelled with ^{125}I (0.5 atoms per molecule). The labelled frattion was then subjected to a final separation on Sephadex G-200. The breakthrough peak, containing the labelled IgM was collected in tubes containing powdered Bovine Albumin (Fraction V, Armour, Eastbourne, Sussex). This separation also removed unbound activity.

For both separations a Pharmacia K100/100 column was used. The initial DEAE bed volume of 72 x 10 cm shrank to 52 x 10 cm. The flow rate over the same period increased from 125 ml/hr to 300 ml/hr. The second separation was carried out using an 82 x 10 cm bed of Sephadex G-200. The flow rate was 120 ml/hr.

Table 16

Third IgM Repeat Study

A.

The Plasma Disappearance (and P.V. Deviation) of IgM preparation (iii), in 2 Calves

Calf No.	P.V. Deviation (%)	Plasma Activity at 50 hrs.	Plasma T½'s (hours) Initial	Final
17	44	8	25	-
18	55	16	58	-

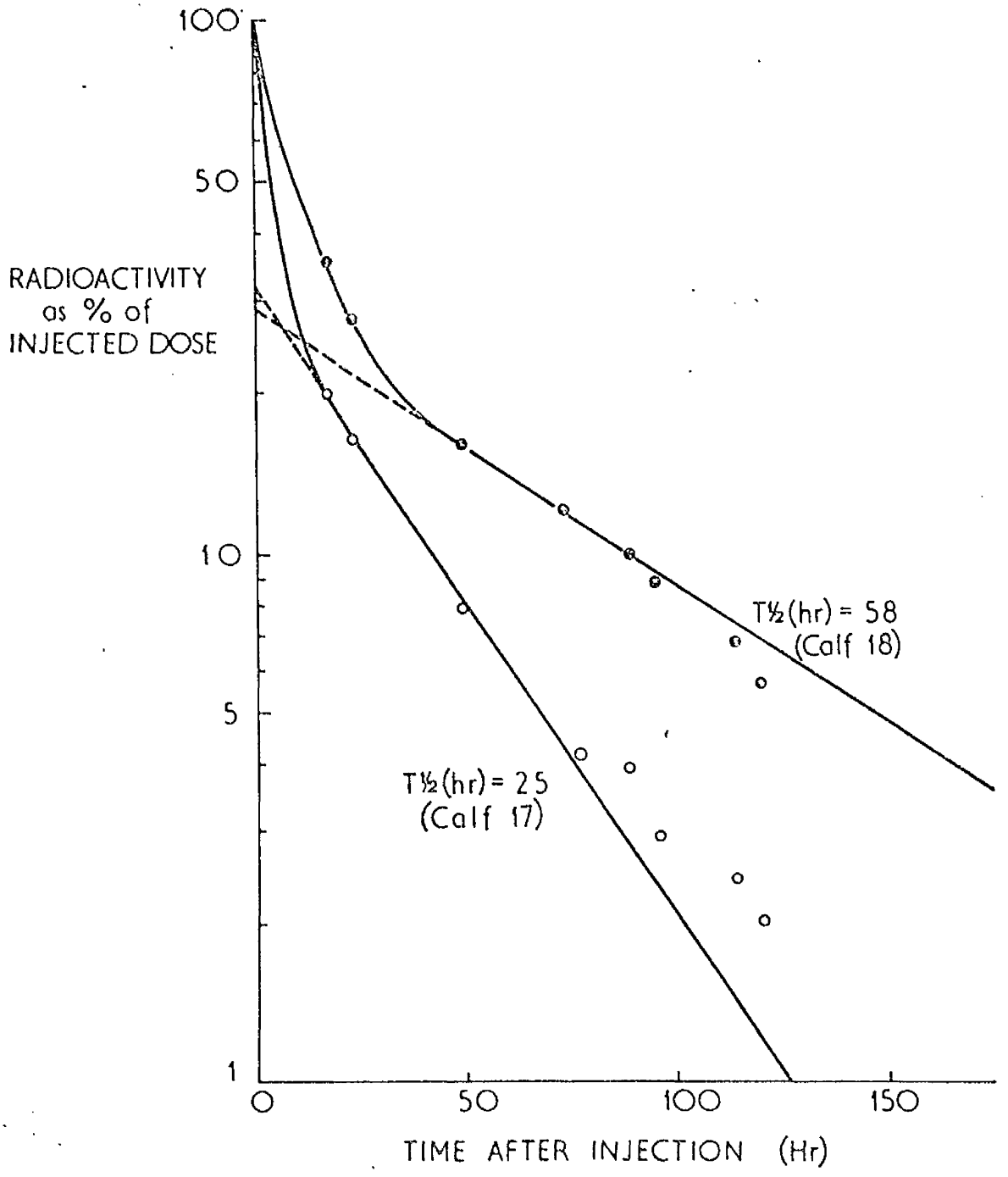
B.

Additional Data for Calves 17 and 18

Calf No.	Mean P.C.V.	Total Protein (gm/100ml)	A/G Ratio	Age (weeks)
17	36	6.7	0.60	1
18	30	7.3	0.46	1

Figure 23

PLASMA DISAPPEARANCE of IgM PREPARATION(III)
in 2 CALVES



For further details about both chromatographic techniques see Section III.

3. Iodination

The number of atoms of iodine incorporated was 0.5 atoms/molecule of IgM.

4. Injection

Each calf received approximately 1 μc of ^{125}I-labelled IgM.

5. For other proceedures, see Section V A.

Results

(see Table 16 and Figure 23)

The plasma volume deviation of approximately 50% was higher than that in the initial study but lower than in the first repeat study. The 50 hour plasma activities and the initial plasma $T\frac{1}{2}$'s were both very low.

This IgM preparation was thus an improvement on no. (ii), but the results indicated a greater degree of denaturation than that present in no. (i).

E. Additional Examination of Preparation (iii) in the Rabbit

To examine the possibility of using rabbits to test a series of IgM preparations, instead of calves, a trial rabbit experiment was carried out, at the same time as the third IgM repeat study, making use of some of the same labelled preparation.

1. Experimental Animals

Three Dutch rabbits weighing 2.0 to 2.8 Kg were used for the experiment. They were housed in metabolism cages (Dargie, 1969) to enable the urine to be collected.

2. Source of IgM

Some of the labelled IgM prepared for Calves 17 and 18 in Section V D was used.

3. Level of Iodination

The number of atoms of iodine incorporated was 0.5 atoms/molecule of IgM.

4. Injection

Each rabbit received approximately 0.6μc of ^{125}I labelled IgM.

5. Plasma Volume Determination

The P.V.'s were calculated from the dilution of the IgM and of Evans Blue, injected simultaneously into the marginal ear vein. Blood samples were taken from the opposite ear at five minutes.

Figure 24

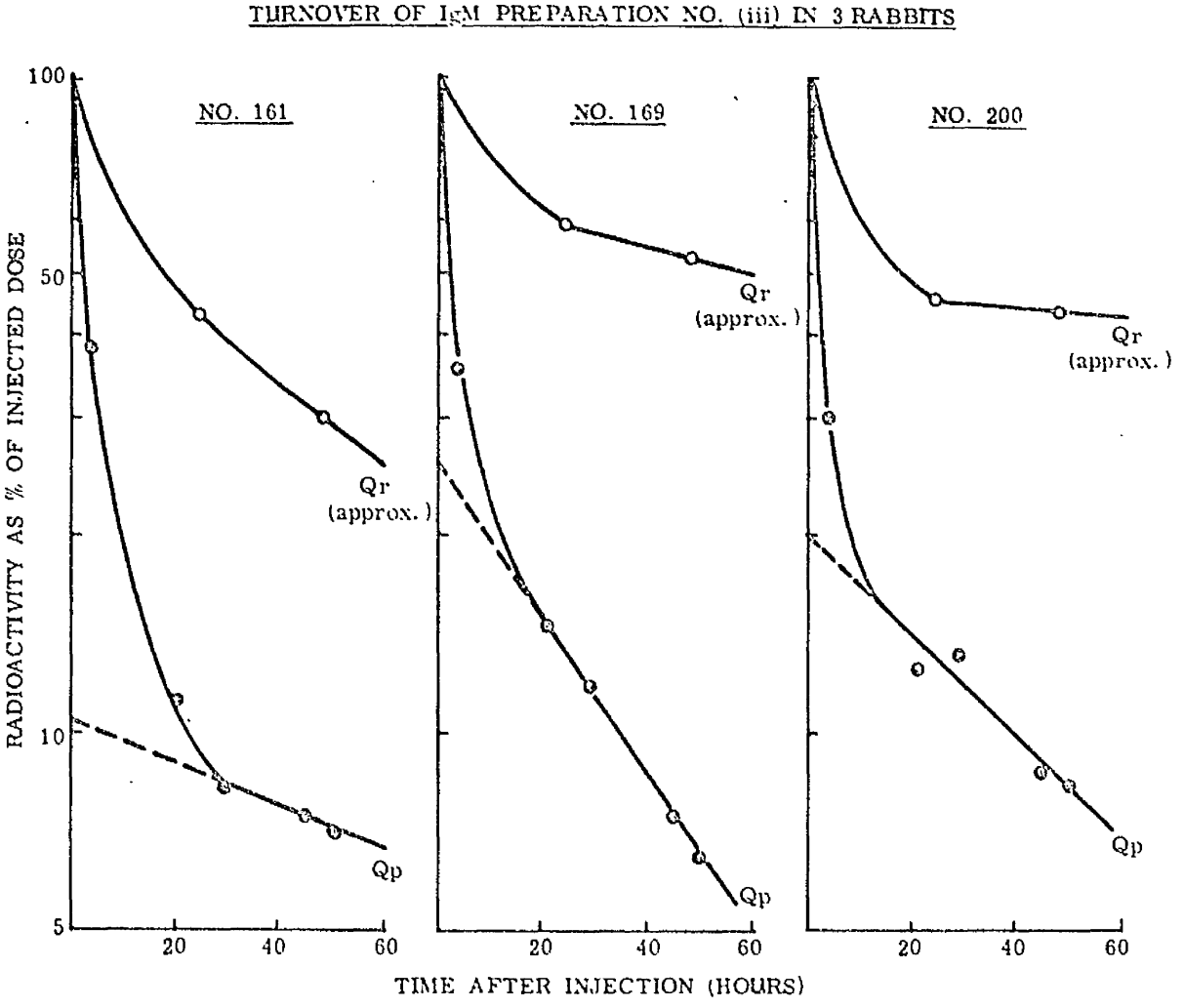

TURNOVER OF IgM PREPARATION NO. (iii) IN 3 RABBITS
NO. 161
NO. 169
NO. 200
Qr (approx.)
Qp
RADIOACTIVITY AS % OF INJECTED DOSE
100
50
10
5
20
40
60
TIME AFTER INJECTION (HOURS)

Table 17

Metabolism of Bovine IgM in the Rabbit
the Denaturation Criteria

Rabbit No.	P.V.Deviation %	Plasma Activity at 50 hours(%)	Urine Activity(%) Day 1	Day 2	Mean P.C.V.
161	-2.2	7.1	56	14	41
169	6.3	6.5	41	5	44
200	19.0	8.4	54	2	35
Mean	3.5	7.3	50	7.0	
S.D.±	13.6	0.97	8.1	6.2	
S.E.±	7.8	0.56	4.7	3.6	

6. Other experimental details

The disappearance of the IgM from the circulation was followed for the first 50 hours and the appearance of activity in the urine in two 24 hour collections. The general management of the rabbits and organisation of the experiment followed the proceedure of Dargie (1969).

Results

(calculated as in the calf experiments)

The plasma acivity was 7.3% ± 0.97% at 50 hours. (see Table 17 and Figure 24). The urinary activity in the first 24 hours was very high, representing 50.3% ± 8.1% of the injected activity. In contrast, only 7.0 ± 6.2% was excreted in the following 24 hours. Moderate agreement was found between the plasma volumes measured with the labelled IgM and with Evans Blue, the difference between them expressed as a %, being 3.5% ± 13.6%.

Conclusions

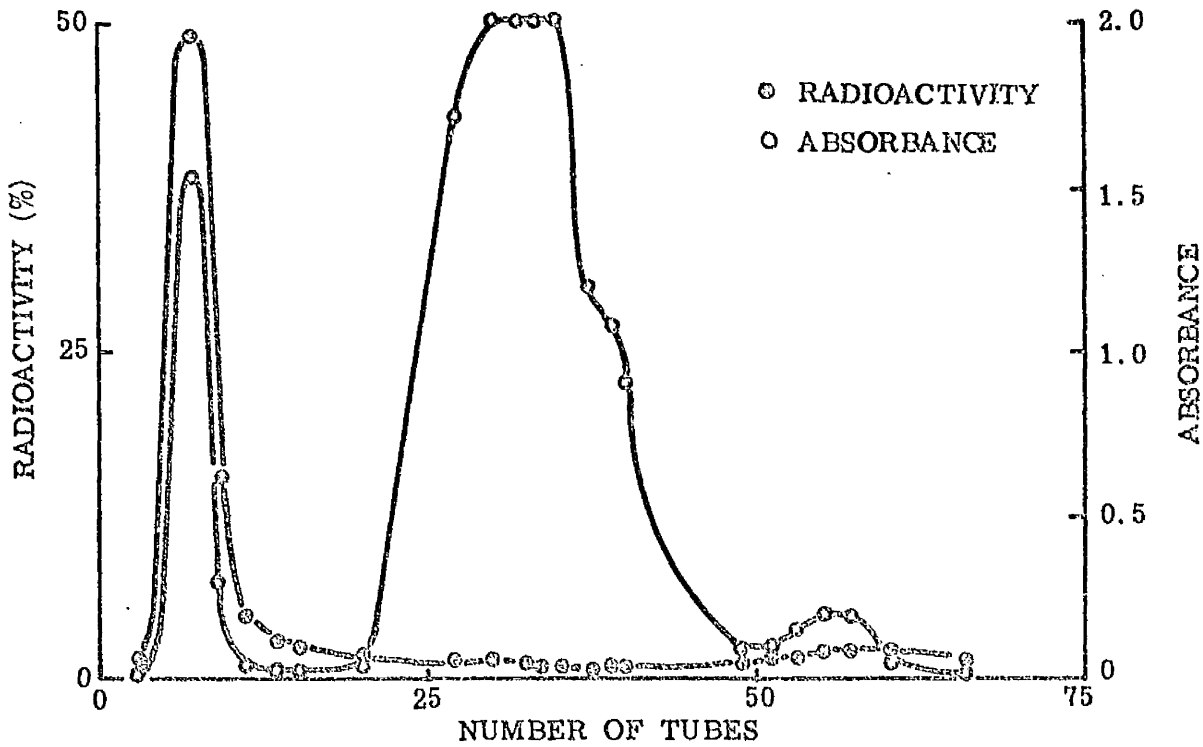
The difference between Day 1 and Day 2 urine activities was significant ($0.01 > p > 0.001$). The low plasma volume deviation of 3.5% in the rabbits must be contrasted with that of 50% in calves 17 and 18, using the same preparation. This

may in part reflect increased capillary permeability of the capillaries of the two calves. The mean 50 hour plasma activity of 7.3 ± 0.97 % is of the same order as that of the two calves, 8 and 16 % respectively. These results, while producing additional evidence that the preparation was partially denatured, when compared with the results obtained with the same preparation in C 17 and C 18 show that the short plasma half lives do in fact reflect this denaturation, and not hypercatabolism associated with Neonatal Diarrhoea.

As well as avoiding problems associated with diarrhoea, the use of rabbits to screen a series of IgM preparations, has other advantages. An estimate of the excreted activity can be readily obtained. Also, it is possible to test small quantities of IgM preparations, with a low specific activity i.e. preparations that could not be examined in calves. To avoid problems associated with the production by the rabbit of antibodies to bovine IgM, rabbits could be rendered tolerant to bovine plasma proteins at birth. However, in view of the rapid catabolism of the IgM preparations so far examined this is not a problem at the moment.

Figure 25

RECHROMATOGRAPHY OF IgM PREPARATION (iv) - IN 2gm % BOVINE ALBUMIN - ON DEAE SEPHADEX A-50, USING A 0.15-0.5 M PHOSPHATE BUFFER GRADIENT (pH = 8.0)



F. Final IgM Experiment

1. Experimental Animal

Calf 18, now 3 months old, had been retained from the earlier study (Section V D).

2. Source of IgM - no. (iv)

The same basic method of prepartion was followed as for Section V D. However, labelling was delayed till after the G-200 separation. Unbound activity was removed by passage through a Sephadex G-200 column, and more Bovine Albumin added. When rechromatographed on DEAE A-50 Sephadex, the majority of the labelled material came through in the break through peak (see Figure 25) indicating some change since the first DEAE separation. The fractions constituting this peak were combined and used for the experiment, without further concentration.

3. Injection

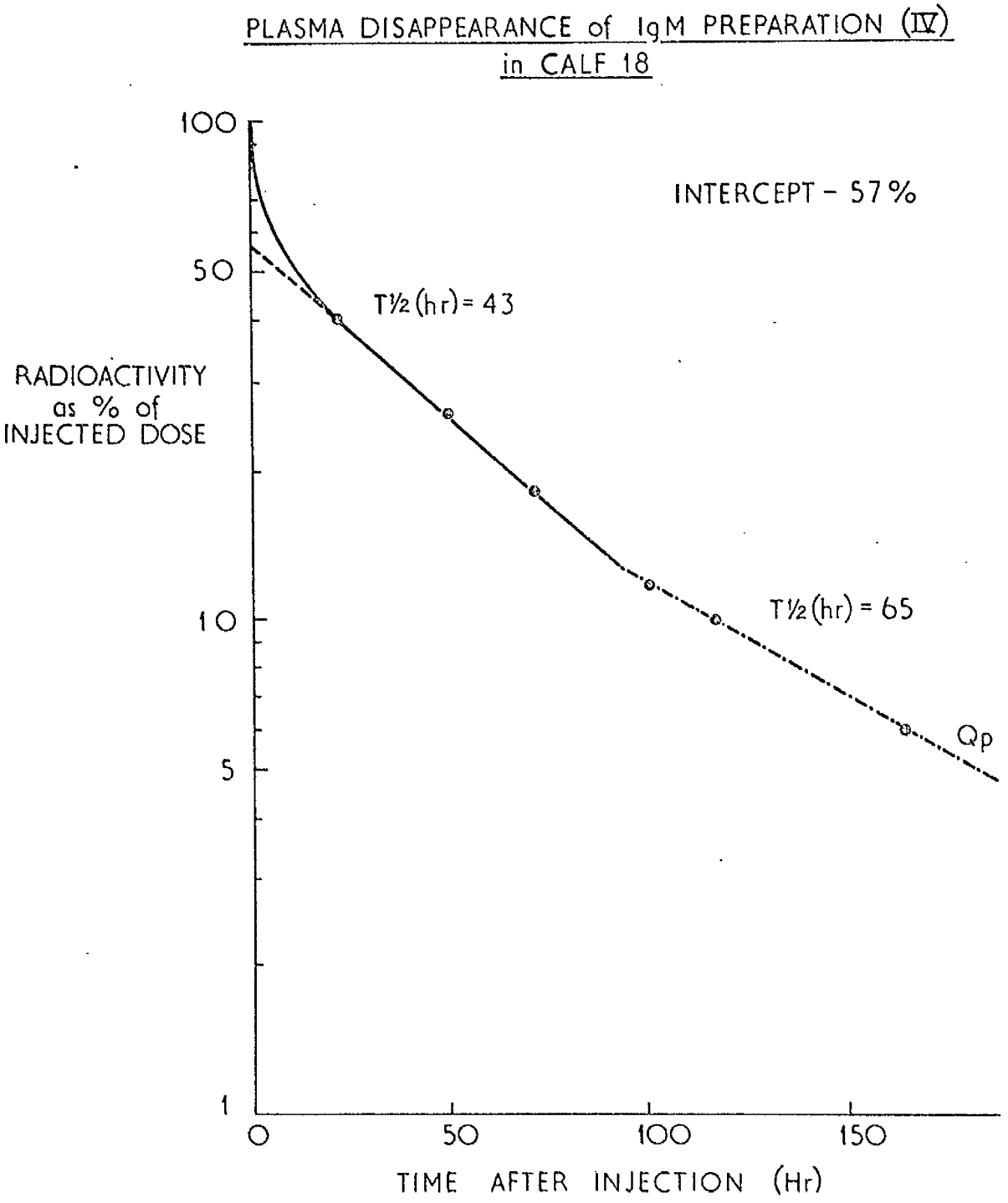
The calf received approximately 1 μc of ^{125}I-labelled IgM.

4. For other proceedures, see Section V D.

Results

The distribution, calculated by the Sterling method was 1/0.75 (I.V./E.V.). The calf had a plasma volume of 39 ml/Kg and a mean P.C.V. of 38. The plasma activity at 50 hours

Figure 26


PLASMA DISAPPEARANCE of IgM PREPARATION (IV)
in CALF 18
INTERCEPT - 57%
T½ (hr) = 43
T½ (hr) = 65
Qp
RADIOACTIVITY
as % of
INJECTED DOSE
100
50
10
5
1
0
50
100
150
TIME AFTER INJECTION (Hr)

was 26% (see Fig. 26). The initial and final plasma $T_{\frac{1}{2}}$'s were 43 hours and 65 hours respectively.

Discussion

The plasma volume was similar to that obtained for C 9 (0.4 atoms/molecule) in Section V C. The 50 hour plasma activity was of the same order as that found in the other studies. In view of the fact that this calf was non-diarrhoeic, the short half-lives suggest that all the molecules were partially denatured, presumable at some point prior to the final DEAE separation. Other workers have considered that rechromatography of IgM on Sephadex G-200 is sufficient to show that the preparation is undenatured. The results here suggest that this is not the case and that ion-exchange chromatography is more sensitive as was suggested by Cohen (1959). The distribution (Sterling) suggests that although the preparation was partially denatured, the degree of denaturation was not sufficient to cause the IgM to be immediately removed from the circulation. This is further supported by the plasma volume.

G. An Estimate of the Plasma Half-Life of Bovine IgM, by Radial Diffusion

1. Preparation of the antiserum

Bovine IgM (contaminated with a trace of α_2 macroglobulin) was prepared by molecular sieve chromatography on Sephadex G-200 (see Section V A 2). Immunoelectrophoresis of the antiserum raised against this preparation (see Section II E 1) showed antibodies against IgM, other immunoglobulins and α_2 macroglobulin. The antibodies "cross-reacting" with the other immunoglobulins were absorbed with IgG_2. To remove the anti α_2 macroglobulin component, it was necessary to prepare the macroglobulin by fractionating adult bovine serum on Sephadex G-200 and then separating the break through peak on Sephadex DEAE A-50. The final antiserum was shown to be pure by double diffusion against IgM fractions and whole serum.

2. Diffusion

(see Section II E 5)

Two plasma samples were examined from four calves, C/C3, C/C6, C/C10 and C/C11 (see Section VI). The first sample had been taken four days post partum and the second, 48 hours later. Adult bovine serum and 3 dilutions of it, were used as relative standards.

Results

When the standards (expressed as d^2) were plotted against the relative IgM concentration, a straight line was obtained. The apparent plasma $T\frac{1}{2}$, for each calf, was then calculated from the change in d^2. C/C3, C/C6, C/C10 and C/C12 had $T\frac{1}{2}$'s of 7.8, 8.4, 6.6 and 5.7 days respectively, giving a mean plasma $T\frac{1}{2}$ of 7.2 ± 1.2 days.

Conclusions

When taken in conjunction with the apparent plasma half-lives of the isotopically labelled IgM preparations, they suggest that the $T\frac{1}{2}$'s obtained in the initial study (see Table 9) were abnormally low and that the Initial $T\frac{1}{2}$'s found for IgM labelled with 0.8 and 0.4 atoms of I/molecule (see Table 14) in older calves, probably give a better indication of the normal rate of disappearance of colostral IgM from the circulation.

Table 18

Comparison of the Denaturation Criteria for The 4 IgM Preparations

Preparation No.	P.V. Deviation (%)	Plasma Activity at 50 hrs.	Day 1/Day 2 Excreted Activity Differences	F.C.R. Variation
(i)	10.3 ± 4.3	25 ± 6.0	Significant	Fairly constant
(ii)	150 ± 27	12.8 ± 3.5	Not Significant	Decreasing
(iii)				
Calves	50 ± 7.8	12 ± 5.7		
Rabbits	3.5 ± 13.6	7.3 ± 0.97	Significant	
(iv) *		26		

* plus abnormal behaviour on ion-exchange chromatography

DISCUSSION

There were certain differences in the methods of preparation of the four IgM fractions. Albumin was not used as a protective colloid in the preparation of (i) and (ii) till after labelling when it was added to reduce the specific activity of the preparation. As already stated in Section V B, the only difference between these two preparations was that no. (ii) was re-chromatographed on G-200 and then stored at -20°C, prior to labelling. By comparison with (ii), preparation (i) was relatively undenatured. It may therefore be assumed that as both had been subjected to chromatography on Sephadex G-200, supposedly a mild proceedure, that the cause of the very marked denaturation of no.(ii) was the storage of the IgM, unprotected by albumin. Although alterations were made in the method of preparation of (iii) and (iv), albumin being added wherever possible, and neither stored at -20°C, preparation (iv) did not bind to DEAE A-50 and neither preparation appeared to be an improvement on no. (i).

It can be seen from Table 9 that the results obtained for the denaturation criteria using the four IgM preparations, varied considerably. In spite of the fact that preparation (i) was

labelled with 1.5 atoms of I/molecule, on the basis of the low P.V. deviation, 50 hour plasma activity and F.C.R.'s, it would appear to be the best preparation so far.

Although many workers must have come up against the problem of denaturation, it has only been investigated and discussed by a few. Thus, protein denaturation was considered at length by Mirsky and Pauling (1936) and by Neurath, Greenstein, Putman and Erickson (1944). It consists of any non-proteolytic modification of the unique structure of the native protein, giving rise to definite changes in its' chemical, physical or biological properties. It is an ambiguous term unless the conditions under which it occurred are clearly established. The degree of denaturation depends on the extent to which the structure is modified. It may result in aggregation, a process not specific for denaturation and it is frequently followed by the related phenomenon of precipitation. However a denatured protein does not necessarily form a precipitate. Their results indicate that certain biological activities require the specific configuration of the whole molecule while others e.g. antibody activity, may not. Denaturation is generally irreversible.

Pirie (1940) considered, in relation to the modification of substances during purification, that difficulties are primarily due to the absence of a satisfactory standard protein.

Colvin, Smith and Cook (1954) found that a relatively small physical difference associated with denaturation could be associated with a profound difference in the biological effect of the protein. Cohen (1959) suggested that the behaviour of a labelled protein on ion-exchange chromatography, was the most sensitive physiochemical test available for danaturation, such denaturation having been caused by self-irradiation, the method of fractionation or the technique of iodination. Freeman (1959) used two in vivo tests, in rats and in rabbits made tolerant to human plasma proteins. Kellar and Block (1960) pointed out that frequently, protein preparations that were believed to be undamaged, have subsequently been found to be at least slightly denatured. The risk of denaturation may be increased by a low salt concentration, removal of protective substances, vigorous stirring, excess of surface active agents. amd, in some cases, freeze drying.

Skvaril (1960) and James, Henney and Stanworth (1964) investigated denaturation resulting from prolonged storage of γ globulin. Andersen (1966) suggested that although a denatured protein may be metabolically heterogenous, this is not necessarly the case. Such an evaluation is more sensitive than physicochemical tests, of which ion-exchange chromatography is probably the best. The metabolic studies may reveal an apparently increased plasma volume, increased excreted activity

in the first 24 hours or a variable Urine/Plasma (U/P) ratio. Freeman (1966) considered that the most common cause of denaturation was the method of isolation. Thus while some of the causes may be well recognised, the corresponding molecular changes are uncertain.

Schultze and Heremans (1966) suggested that any agency which interferes with the structure of the protein, will cause denaturation. Resistance to denaturation will therefore depend on the stability of this structure. Clausen (1969) showed that Immunoelectrophoresis may detect denaturation, e.g. from over iodination. Freeman and Smith (1970) working with Sephadex G-150, considered that some of their results could be explained in terms of denaturation caused by polymerisation, loss of peptides or change of configuration. Freeman (1970) considered that when a new protein was being examined, it was difficult to be certain that the material under study was not all equally altered. Nielsen and Nansen (1970) demonstrated the effect of self-irradiation on ^{131}I-labelled immunoglobulin in sheep.

While the risk of denaturation has to be borne in mind for all metabolic plasma protein studies, it can be seen from the work on human IgM that it is a particular problem with this immunoglobulin. Muller-Eberhard, Kunkel and Franklin (1956) found that they were never able to completely eliminate larger molecules from their 19s preparation. This tendency to form aggregates when purified was also noted by Schultze, Haupt,

Heide, Moschlin, Schmidtberger and Schwick (1962). After repeated ultracentrifugation they found that only a minor 19s component was present. Wilkinson et al (1966) suggested that the initial rapid decline in plasma and body levels of their labelled macroglobulin might be accounted for by an aggregated fraction.

Deutsch (1967) found 15 to 35 % of his IgM preparation consisted of polymers. Jensen (1969b) made a similar suggestion with regard to homologous IgM studies. Alternatively, partial reduction of IgM might be the cause. Becher and Storiko (1970) showed that standardsprepared for radial diffusion might contain only 43% of 19s molecules. As well as this tendency to polymerise, Mannik (1967) showed that γM forms complexes with albumin, in vivo.

There is evidence that some of the first IgM preparetions used for metabolic studies were denatured, to variable degrees. Thus Cohen and Freeman (1960) obtained an initial Fractional Catabolic Rate (F.C.R.) of 35 to 38 %/day which decreased to 15 to 18 %/day after 5 to 7 days. Olesen (1963) found that 3 out of 7 of his IgM preparations were partially denatured, in spite of the use of mild methods of preparation. He showed that a single euglobulin precipitation may cause a gross denaturation and even a cold agglutinin preparation was partially

denatured. Svartz, Schatz and Hedman (1965) found that although cold fractionation did not appear to alter Rheumatoid Facter ammonium sulphate precipitation dissociated it and changed the characteristic Immunoelectrophoresis pattern. Wilkinson et al (1966) considered that macroglobulins were easily denatured during purification, particularly if repeatedly precipitated and separated by ultracentrifugation.

Birke et al (1967) could detect no physico-chemical changes in their IgM preparations, by electrophoresis, immuno-electrophoresis or molecular sieve chromatography, as previously suggested by Plantin and Norberg(1965). They were unable to rule out partial denaturation in a few of their patients, who showed an increased elimination in the first few days. They considered that there was some doubt about earlier IgM studies. Jensen (1969a) concluded that his IgM preparations were either partially denatured or heterogenous. Olesen and Hippe (1969) found that molecular fragments were catabolised at a much higher rate. During purification IgM was relatively easily denatured, in part or total.

Jensen (1969b) showed that the method of Barth et al (1964) and the use of DEAE cellulose combined with Sepharose 4 B gel filtration both caused partial denaturation of IgM. He found that initial lipo-protein removal followed by molecular

sieve chromatography on Sepharose 4 B, before and after isotopic labelling gave a superior separation. For the second separation the IgM was protected by donor serum. Norberg et al (1970) had similar problems with α_2 macroglobulin. Their labelled preparation still contained 15 to 20 % of 15s material, but found that recycling increased the risk of fragmentation.

It is thus hardly surprising that the first Bovine IgM studies described here have run into the problem of denaturation. In addition, no allowance was made for aggregation other than rechromatography on Sephadex G-200. While aggregation does not in itself necessarily constitute denaturation it is often associated with it. It is one possible explanation of the denaturation occurring during the preparation of IgM and may in part account for the abnormal behaviour of preparation (iv) on DEAE A-50 Sephadex. In future it would be advisable to examine all preparations by ultracentrifugation, before use. This tendency for IgM to aggregate has been noted before, by a number of workers (see Introduction). The breakdown of the 19s units to smaller units would have been detected by rechromatography. Thus fragmentation can be ruled out as a major source of the denaturation.

In the earlier work with IgM in man it was found necessary to wait until the daily F.C.R. became constant. Such a preparation

while yielding information about the metabolism of IgM would still be unsatisfactory for determining the efficiency of absorption of colostral IgM.

Various suggestions have been made as to how the problem of denaturation may be limited or avoided. Alexander and Block (1960) suggested that the risk of protein denaturation could be reduced, by a high concentration of neutral salts, a pH close to the isoelectric point, processing and storage at low temperatures and the presence of lipids.

Killander and Philipson (1964) abandoned the use of the Tris-NaCl buffer for molecular sieve chromatography of Rheumatoid Factor, in favour of a phosphate buffer. Porath and Ui (1964) prepared γ globulins using glycine rich solvent systems. The use of glycine to stabilise solutions of γ globulin had been suggested previously by Kabat and Meyer (1961). Porath and Ui also included ε- amino - caproic acid to prevent proteolytic digestion. (as used by Skvaril and Grunberger, 1962). Other workers have suggested the addition of albumin, dextran or whole serum to purified or partially purified preparations (Ahlinder et al, 1965, and Plantin and Norberg, 1965). Finally, Jensen et al (1970) suggested that some of the difficulties associated with IgM separation proceedures, may be partially overcome by the use of serum

with a high content of monoclonal IgM, as starting material.

In view of these suggestions and of the findings in these studies, a number of points should be considered before further investigations are undertaken. If the basic G-200 separation is retained, the following changes should be considered. A change of buffer, from Tris-HCl to a Phosphate or Glycine buffer. The use of albumin as a "protective colloid" which was started in these studies, or alternatively, fibrinogen or a high molecular weight dextran.

Alternatively, G-200 could be replaced by Sepharose 4 B as used by Jensen (1969). Another method worth considering would be large scale iso-electric focusing (L.K.B., Croydon, Surrey). Ion-exchange chromatography should probably not be used to prepare IgM for metabolic studies because of a risk of denaturation resulting from unfolding of the molecule.

Whatever basic method of preparation is used, where possible fresh as opposed to stored serum or colostral whey should be used as starting material and during preparation, storage at -20°C should be avoided.

In addition to rechromatography on DEAE A-50 and G-200 Sephadex the preparations should be examined by analytical ultracentrifugation. In-vivo examination in rabbits made tolerant to bovine plasma proteins would also be very useful.

Finally, with regard to the experimental animals, where possible it would be better to avoid the use of diarrhoeic calves, until control values have been established.

SUMMARY

Four preparations of Bovine IgM (obtained by molecular sieve chromatography) were studied. Evidence was found which indicated that they were all partially denatured and thus not suitable for providing the information required for a study of the absorption of colostral IgM. Suitable denaturation criteria were investigated. The distribution of two of the preparations indicated that Bovine IgM (as in other species) is retained to a greater extent within the circulation, than IgG. Although apparent plasma half-lives varied considerably, V B, C and G all indicated a $T_{\frac{1}{2}}$ of between 5 and 10 days.

SECTION VI

THE EFFICIENCY OF ABSORPTION OF COLOSTRAL FAST IαG($IαG_1$)

INTRODUCTION

The importance of colostrum in the transfer of passive immunity was recognised by Ehrlich (1892) along with placental transfer, in the mouse. Famulener (1912) showed that in goats, the role of the placenta was negligeable. He considered that the main difference between colostrum and milk was in the amount of serum proteins present, normal milk containing only a trace of globulin. The high antibody levels in colostrum resulted from a storage of antibodies removed from the blood stream. These antibodies were most readily absorbed immediately after birth, age being critical in relation to ability to absorb. Subsequent work has expanded these ideas, and applied them to other ruminants.

Howe (1921) demonstrated that the calf receives its' first supply of globulins from the colostrum. After the ingestion of colostrum relatively large amounts of euglobulin and pseudoglobulin I were detected in the serum of the calf. Smith and Little (1922) in a controlled experiment, demonstrated the importance of colostrum in ensuring the survival of the newborn calf. They concluded that the calf deprived of colostrum lacks something. This situation permits intestinal bacteria to invade the body and multiply in the various organs.

Aschaffenburg, Barlett, Kon, Walker, Briggs, Cotchin and Lovell (1949) showed that the protective factor in colostrum was present in the aqueous phase. They produced additional evidence for the relationship between the ingestion of colostrum by the newborn calf and subsequent survival.

Dixon, Weigle and Vazquez (1961) showed that the amount of γ globulin in the colostrum corresponded approximately to the amount lost from the circulation of the cow. Little or no γ globulin, was formed in the normal udder. The acinar epitholium acts primarily as a transporter of serum protein, which is transfered unchanged. Hammer, Kickhofen and Machow (1968) considered that this transfer, without obvious degradation and resynthesis, was one of selective transport and secretion of Fast IgG. The selection of Fast rather than slow IgG, they suggested, depended on either a specialised transport function of the Fast IgG molecules or specific receptor sites on the surface of the acinar cells corresponding to sites on the Fc fragment of the Fast IgG molecule. They demonstrated the specifity of the process and found some evidence to support the concept of specific receptor sites. Subsequently, Fey (1971) found evidence that the Fc piece does not play a decisive role.

The relationship between hypogammaglobulinaemia in neonatal

calves and the absorption of colostral immunoglobulins, has been studied by a number of workers. Selman (1969) demonstrated the importance of management factors. When these are eliminated, the level of Colostral Immunoglobulin becomes important. Out of 120 calves, not one showed decreased absorptive ability. Some of the factors influencing the quality and quantity of bovine colostrum were investigated by Kruse (1970a). He found a wide variation in the total yield of Immunoglobulin (Ig) at the first milking, after calving. It reflected the Ig concentration at earlier calvings, older cows giving better colostrum, and no seasonal variation was apparent. He considered that 12% of his sample provided an inadequate amount of Ig. From computer simulation studies, (Kruse, 1970c) he concluded that hypogammaglobulinaemia cannot be completely avoided under reasonable farming conditions, because of variations in birth weight, amount of colostrum offered and the age of the calf at first feeding.

While many workers have measured the absorption of specific colostral antibodies or the colostral immunoglobulins in terms of plasma levels, it has not been possible, until the studies in Section IV were completed, to determine with any certainty, the <u>total</u> absorbed. Some workers in fact have ignored the fact that a considerable proportion of the colostral IgG_1

absorbed by the neonatal calf will be distributed extravascularly i.e. in the extravascular compartment (see Section IV, Introduction). Other workers have recognised that it is essential for the quantitation of absorption, particularly in terms of the efficiency of the mechanism, for the size of this extravascular compartment to be known. Once established, a variety of methods can be applied to determine the quantity of a particular antibody or immunoglobulin in the colostrum and in the plasma (or serum) of the neonatal calf. To complete the quantitation, it is only then necessary to determine the plasma volume at the time at which the plasma level is assessed. This may be performed with Evans Blue or with Albumin or IgG, labelled with the isotopes of iodine. The timing of this plasma volume measurement is important. Selman (1969) based his 48 hour plasma sampling time on the time taken for γ globulin to equilibrate in the rabbit. Kruse (1969) on the other hand used a 24 hour sample, when plasma levels may be maximal. In Section IV the apparent equilibrium time for IgG had been found to be 45 ± 16 hours. Thus, allowing for the interval between parturition and feeding, the delay between the ingestion of colostrum and the appearance of significant quantities of IgG_1 in the plasma and also for the period of absorption itself, the time for measuring the plasma volume and plasma level was fixed at 48 to 72 hours.

MATERIALS AND METHODS

A. Source of Calves

All but one of the calves were obtained from Gartloch Farm, Gartcosh, Baillieston (Calf C/C8 from Woodilee Farm, Lenzie). The calves were less than three hours old and had been removed from their dam at birth. With the exception of C/C1 (an Ayrshire heifer calf), they were all Ayrshire bull calves.

B. The Colostrum

All but one of the calves received maternal colostrum. C/C6 received the same colostrum as C/C3 which had been stored at -20°C until required. A tracer quantity (approx. 400 μc) of ^{131}I labelled Fast IgG, prepared in advance and stored at -20°C was added to a known volume of the colostrum that the calf was to receive. 5 or 15 ml colostral standards were prepared and 20 ml of colostrum retained for further examination. The temperature of the colostrum and of the small quantities of milk given before and immediately after it, was approximately 20°C.

C. Feeding of Colostrum

(3 to 6 hours post partum)

C/C2 was fed using a Rose-Miller funnel. All the other

calves were fed a fixed quantity of colostrum by stomach tube. Prior to feeding, the calves were dried and weighed. Where urine and faeces were to be collected a brisket harness, "rubber chute" and polythene bag were attached to the calf which was then placed in a metabolism cage (see Section II B 2b).

A stomach tube (Foal size- Portex Ltd., Hythe, Kent) with funnel attached, was passed via the nose, so that the end of it was approximately half way down the oesophagus. Suckling movements were then stimulated by feeding the calf 20 ml of milk from a syringe. Finally, before giving the colostrum, 50 ml of saline was poured into the tube to check its position, as far as was possible. With the funnel raised to a suitable height the colostrum was slowly administered. The tube was washed out with 50 ml of milk before being removed.

D. Administration of Iodine

Where an attempt was made to measure the total loss of ingested IgG, necessitating the collection of all excreted activity over the first 48 to 72 hours, a balanced solution of KI/NaI (containing the equivalent of 1 gm of I) was slowly injected via the intravenous catheter (see Section II C 1b). An oral dose of KI was administered at the same time and subsequently every 24 hours.

E. Blood Samples

To determine the rate of appearance of IgG in the plasma, samples were taken every 10 to 15 minutes over a 2 hour period, once significant levels had been detected.

F. Subsequent Feeding

The first milk feed was given 6 to 12 hours after feeding colostrum. Milk was then fed twice daily, see Section II A 2.

G. Efficiency Determination

48 to 72 hours after feeding colostrum, the plasma volume was measured with ^{125}I-labelled IgG (approx. 1 mc/calf). Using this plasma volume and the Extravascular/Intravascular ratio of 1.2/1 (from Section IV) the total body activities of the ^{131}I IgG$_1$ was calculated from a plasma sample taken immediately prior to injection of the ^{125}I labelled IgG. This activity was then expressed as a % of the total activity in the colostrum fed. The efficiency of absorption of IgG thus determined will be subsequently referred to as the Isotopic Efficiency.

The IgG concentration in the pre-colostral, 48 to 72 hours serum samples and colostral whey samples were determined by Radial Diffusion (Section II E 5), using an anti-bovine IgG antiserum (adsorbed with bovine IgM). Standards were prepared from IgG$_2$ (supplied as 7s - Gamma Globulin, 100% pure by Immunoelectrophoresis, from Mann Research Laboratories, New York).

The total amounts of colostral IgG (gm) in the colostrum and in the calf at 48 to 72 hours were determined using a correction factor of 0.841 for the colostral IgG, to allow for the casein clot (Selman, 1969). The Radial Diffusion Efficiency was then obtained from the ratio of IgG absorbed/IgG fed and expressed as a %.

H. Distribution and Catabolism of IgG_1

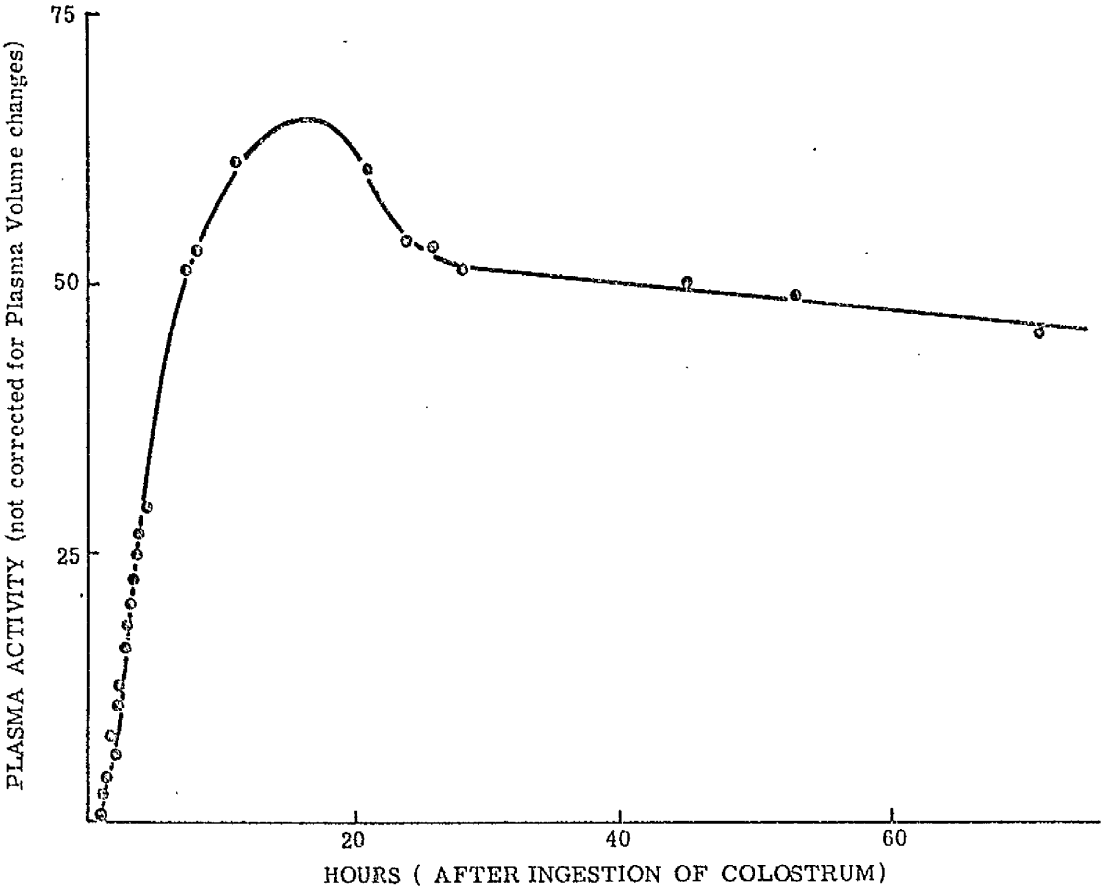
In 12 of the calves, the distribution (by one or both methods, see Section IV) and the catabolism of the IgG_1, used for the 48 to 72 hour plasma volume were studied, as in Section IV.

I. Other Experimental Details

These have already been covered in Sections II, III and IV.

Figure 27

THE APPEARANCE OF ^{131}I-LABELLED FAST IgG IN THE PLASMA OF C/C3
(EXPRESSED AS A % OF THE TOTAL ABSORBED)



RESULTS

A. Initial Appearance

The appearance of the tracer was followed for the first 48 to 72 hours, after the ingestion of colostrum. In five of the calves significant levels of ^{131}I-labelled IgG were detected at 2.2 ± 0.9 hours. As it was not possible to collect regular samples over the whole period in every case, this may not represent the earliest time for all the calves. For C/C3 and C/C4 the initial rise was found to be gradual. This was followed in every case by a steep rise in the level of activity representing the predominance of absorption over distribution. Initially, in three of the calves (C/C3, C/C5 and C/C11) the plasma activity doubled every 0.40 ± 0.11 hours. This then decreased to every 1.4 ± 0.25 hours (C/C3, C/C4, C/C5, C/C6 and C/C7). The apparent rate of absorption was thus changing all the time. As absorption decreased and/or distribution predominated, the rate of increase decreased still further (see Fig. 27). In four calves (C/C3, C/C4, C/C6 and C/C11) the highest plasma activity was reached 18 ± 5.4 hours after the initial ingestion of colostrum. It finally appeared to level out at 29 ± 11 hours, as catabolism becomes the over-riding process. Initially, the plasma activity represents

Table 19

Efficiency of Absorption of Colostral Fast IgG(IgG_1)

Calf No.	Volume of Colostrum (litres)	Isotopic Efficiency (%)	Radial Diffusion Efficiency (%)
C/C1	1.00	81	69
C/C2	2.00	44	61
C/C3	4.00	40	57
C/C4	2.38	49	58
C/C5	4.00	23	26
C/C6	1.00	49	79
C/C7	1.00	57	60
C/C8	4.00	59	66
C/C9	4.00	27	40
C/C10	1.00	32	53
C/C11	4.00	31	20
C/C12	1.00	42	59
C/C13	1.00	65	56
Mean		46	54
S.D.±		16.5	16.5
S.E.±		4.6	4.6

the interaction between absorption, distribution and catabolism, the role of absorption subsequently decreasing, followed by that of distribution.

B. "Isotopic" Efficiency

(see Table 19)

The mean efficiency of absorption for all 13 calves was 46 ± 16.5 %. When the results are broken down on the basis of the volume of colostrum fed (calves C/C2 and C/C4 excluded) this represented 54 ± 17 % for Group 1 (1litre) and 36 ± 14 % for Group 2 (4 litres). The calves were also divided into groups on the basis of which efficiency range they corresponded to.

Efficiency Range	Calves
0 - 10	None
10 - 20	None
20 - 30	C/C3 C/C9
30 - 40	C/C11 C/C10
40 - 50	C/C2 C/C4 C/C5 C/C12
50 - 60	C/C7 C/C8
60 - 70	C/C13
70 - 80	None
80 - 90	C/C1
90 - 100	None

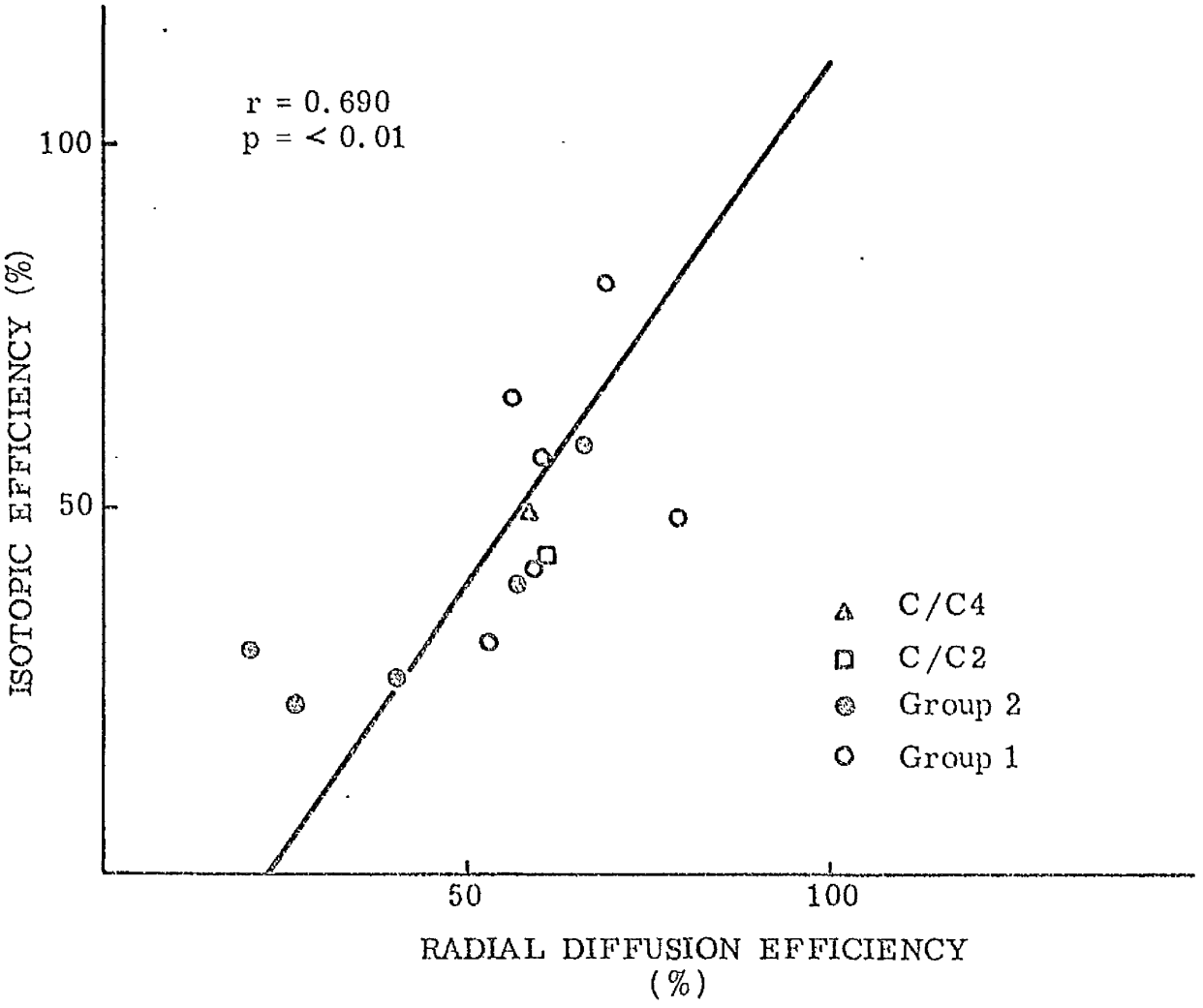
Table 20

IgG Levels in Colostrum and Plasma and Total Quantities (gm) of IgG fed and absorbed (in 13 calves)

Calf No.	Colostral IgG Level (mg/ml)	Total IgG fed (gm)	Plasma IgG Level (mg/ml)	Total IgG Absorbed (gm)
C/C1	72	61	8.0	42
C/C2	77	130	18	79
C/C3	83	279	30	159
C/C4	142	285	32	164
C/C5	137	460	25	118
C/C6	83	70	11.1	55
C/C7	86	73	8.7	43
C/C8	70	234	29	155
C/C9	86	291	26	117
C/C10	106	89	11.0	47
C/C11	83	279	10.6	55
C/C12	51	43	7.3	25
C/C13	33	27	3.0	15
Mean	85	179	16.9	83
S.D.±	30	134	10.1	53
S.E.±	8.3	37	2.8	15

Figure 28

THE CORRELATION BETWEEN THE ISOTOPIC EFFICIENCY AND THE RADIAL DIFFUSION EFFICIENCY



This shows that although the numbers were small, there was a tendency towards a normal distribution.

C. Radial Diffusion Results

The total amounts of IgG absorbed are shown in Table 20, along with the concentrations (mg/ml) of IgG in the colostrum and plasma at 48 hours to 72 hours, from which the total quantities were calculated.

Low pre-colostral levels (<1.0 mg/ml) were detected in only three calves, C/C1, C/C5 and C/C6. IgG was not however detected by immunoelectrophoretic examination of these samples. The precision of the radial diffusion method is of the order of 10%. (Penhale and Christie, 1969). The pre-colostral levels were thus not subtracted from the 48 to 72 hour IgG levels, when calculating the Efficiency of absorption.

The mean Radial Diffusion Efficiency was 54 ± 16.5 . For Group 1 this represented 63 ± 9.6 % and for Group 2 (4 litres) 42 ± 19.7 %. The comparison between the two methods for calculating the Efficiency can be seen in Table 19 and Figure 28. The positive correlation ($r = 0.691$, $p = < 0.01$) is highly significant.

When the total amount of IgG (gm) absorbed by each calf was plotted against the total fed (see Fig. 29) very significant positive correlations were found for all 13 calves (i) and Group 1 (ii), the probability (p) being greater than 0.01

Figure 29

THE CORRELATION BETWEEN THE TOTAL IgG (gm) ABSORBED AND THE TOTAL IgG(gm) FED

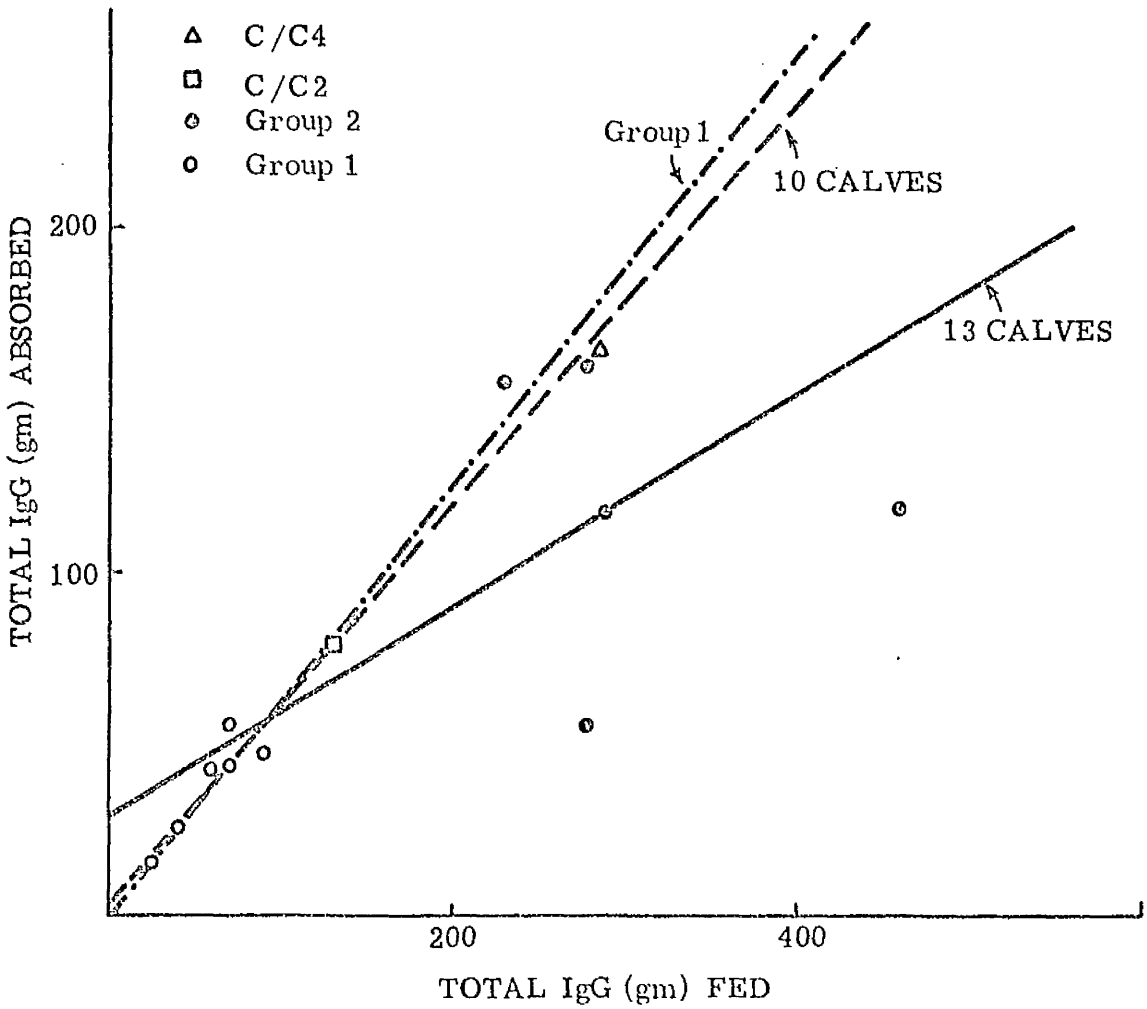


Figure 30

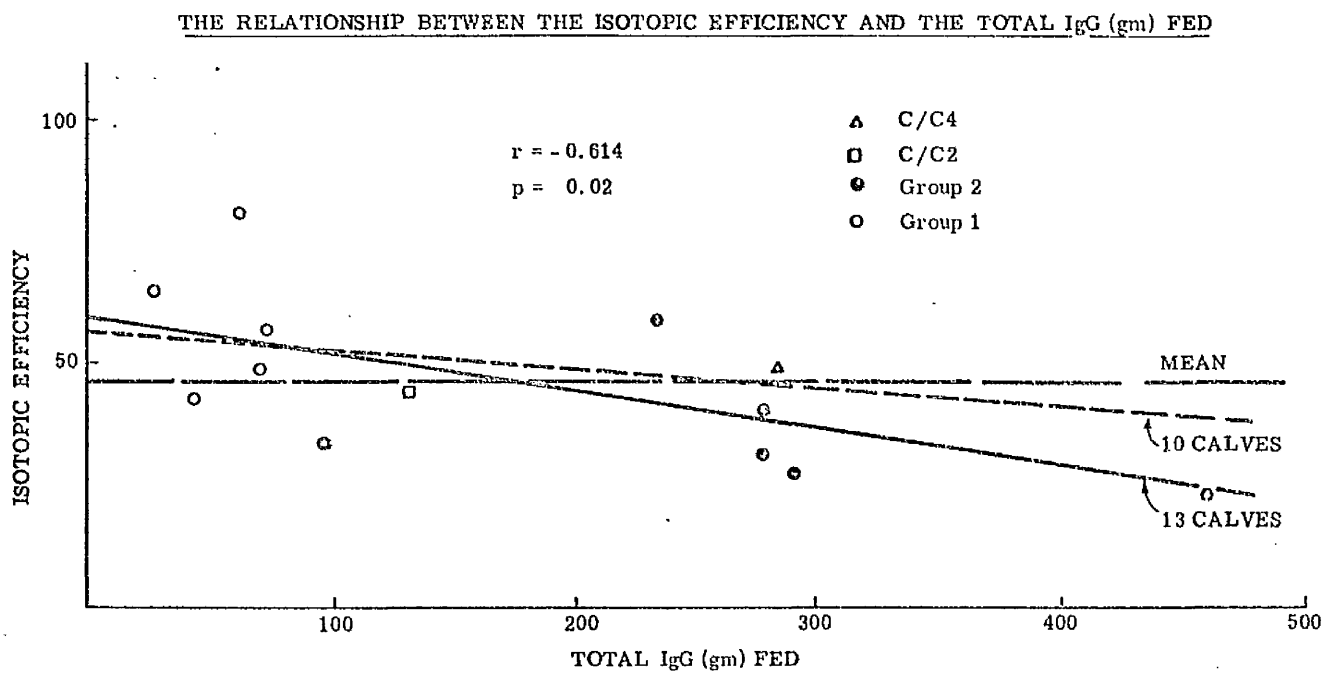
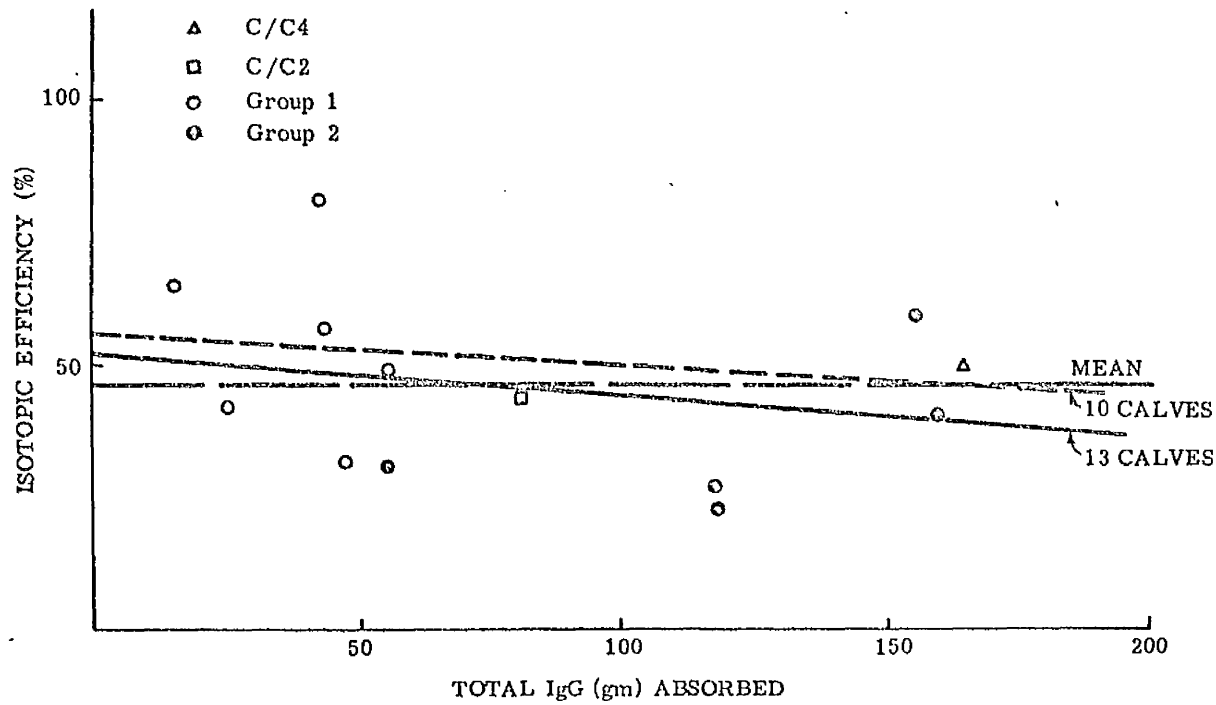

THE RELATIONSHIP BETWEEN THE ISOTOPIC EFFICIENCY AND THE TOTAL IgG (gm) FED
100
50
ISOTOPIC EFFICIENCY
r = - 0.614
p = 0.02
C/C4
C/C2
Group 2
Group 1
MEAN
10 CALVES
13 CALVES
100
200
300
400
500
TOTAL IgG (gm) FED

Figure 31

THE RELATIONSHIP BETWEEN THE ISOTOPIC EFFICIENCY AND THE TOTAL IgG (gm) ABSORBED



but less than 0.001. When calves C/C5, C/C9 and C/C11 from Group 2 were excluded (iii) the probability became less than 0.001. All three regression lines pass close to the origin, particularly (ii) and (iii), suggesting that the ratio of the quantities absorbed and offered i.e. the efficiency, is virtually a constant. For (ii) and (iii) this would be 60%.

When the Isotopic Efficiency was plotted against the total amount of IgG fed (see Fig 30) the calculated regression lines for 13 and 10 calves (as above) indicated basic "initial" Efficiency values of 60% and 57% respectively. There was a negative correlation between the Isotopic Efficiency and the total offered, ($r = -0.614$, $p = 0.02$) for the 13 calves. Similarly when the Isotopic Efficiency was plotted against the total amount absorbed (see Fig. 31), the regression lines indicated an "initial" Efficiency of 52% and 56% respectively. Neither of the regression coefficients were significant.

By comparison with the IgG levels (mg/ml) in colostrum found by other workers, 43.3 ± 14.0 (Klaus et al, 1969) and 34.1 ± a S.E. of 2.1 (Penhale and Christie, 1969) the mean level of 85 ± 30 mg/ml in these studies (see Table 20) is high. The mean plasma IgG level of 16.9 ± 10.1 is considerably lower than 31.6 ± 13.6 found at 48 hours in suckled calves by Klaus et al (1969) but

is higher than 7.5 ± a S.E. of 1.0, found by Penhale, Christie, McEwan, Fisher and Selman (1970) in surviving market calves.

D. First Three Days Excreted Activity

Table 21 shows the apparent distributions of the ^{131}I IgG_1, in four of the calves. These were calculated from the ratio of the total plasma activity to the apparent retained activity less the total plasma activity at 72 hours. At this time there was an apparent distribution (E.V./I.V.) of 1.7/1 ± 0.22. The same table shows the urine protein bound activity (expressed as a % of the activity in the colostrum) which gradually increased over this period.

E. Other Results

Tables 22 to 24 show the results obtained from the additional metabolic studies carried out with the IgG_1 used for the 48 to 72 hour plasma volume.

F. Diarrhoea

Four of the calves, C/C4, C/C5, C/C7 and C/C11 died after 3 to 5 days diarrhoea (+++), 16, 10, 13 and 14 days post partum, respectively. Over the same period (17 days) a further four calves, C/C1, C/C2, C/C8 and C/C9 were also diarrhoeic (+++ for 3 to 5 days).

Table 21

The Apparent Distribution of Labelled Colostral IgG_1 at 48-72 hours and the Urine Precipitates (as % of the Total Activity in the Colostrum)

Calf No.	Calculated Distribution (E.V./I.V.)	Urine Precipitates			
		Day 1	Day 2	Day 3	Mean
C/C6	1/1.5	0.23	0.62		0.43
C/C7	1/2.0				
C/C9	1/1.6	0.79	0.62		0.71
C/C11	1/1.7	0.13	0.30	1.6	0.68
Mean	1/1.7	0.38	0.51		0.61
S.D.±	/0.22	0.35	0.18		0.15
S.E.±	/0.10	0.20	0.10		0.08

Table 22

The Distribution (by Campbell and Sterling methods) and the Catabolism of Labelled IgG_1 in six of the Colostrum fed Calves

Calf No.	E.V./I.V. Sterling Ratio	E.V./I.V. Campbell Ratio	Apparent Equilibrium Time (hours)	Total Body Catabolic Rate	Fractional Catabolic Rate
C/C4	2.0/1	1.3/1	48	0.051	0.131
C/C5	1.5/1	1.2/1	36	0.051	0.089
C/C6	2.0/1	1.2/1	48	0.047	0.114
C/C7	1.5/1	1.3/1	60	0.047	0.103
C/C9	1.5/1	1.4/1	60	0.095	0.173
C/C11	1.8/1	1.8/1	84	0.038	0.104
Mean	1.7/1	1.4/1	56	0.055	0.119
S.D.*	0.25/	0.22/	16.4	0.020	0.029
S.E.*	0.10/	0.09/	6.7	0.008	0.012

Table 23

Half-Lives and additional data for Calves in Table 22

Calf No.	P.V. (ml/Kg)	Apparent Plasma $T\frac{1}{2}$	Total Body $T\frac{1}{2}$	Mean P.C.V.	P.V./Body Weight (%) change over 14 days
C/C4	68	55	12.2	24	
C/C5	52	35	14.4	33	
C/C6	68	22	16.0	28	1.3/4.5
C/C7	59	13.1	19.0	33	
C/C9	69	17.8	7.1	31	26/
C/C11	71	14.6	18.7	46	
Mean	65	26.3	14.6	33	
S.D.±	7.4	16.1	4.5	7.5	
S.E.±	3.0	6.6	1.8	3.0	

Table 24

The Distribution (Sterling) and Catabolism of Labelled IgG_1 in a further six Colostrum fed Calves (plus additional data)

Calf No.	E.V./I.V. Sterling	Apparent Plasma $T\frac{1}{2}$	P.V. ml/Kg	% changes over 14 days P.V.	% changes over 14 days Body Weight	Mean P.C.V.
C/C1	1.2/1	14.8	66		-21	24
C/C2	1.4/1	9.8	64		- 6	31
C/C3	1.1/1	17.8	58		2	36
C/C8	1.4/1	14.1	62	7	7	34
C/C10	1.4/1	12.4	60	18	23	35
C/C12	1.5/1	18.2	52	4	1	30
C/C13		16.6	71			41
Mean	1.3/1	14.8	62		1.0	33
S.D.±	0.15/	3.0	6.1		14.5	5.4
S.E.±	0.05/	1.1	2.3		5.9	2.0

Table 25

Plasma Protein Results, expressed as Albumin (IID 3b) and Globulin concentrations (gm/100ml) for all 13 Colostrum fed Calves

	(Precolostral)		(48-72 hours)		(10 days)		(17 days)	
Calf No.	Alb.	Glob.	Alb.	Glob.	Alb.	Glob.	Alb.	Glob.
C/C1	1.9	2.8	1.9	3.6	1.9	3.9	2.2	4.4
C/C2	1.7	1.8	1.6	3.6	1.8	3.4	1.5	2.8
C/C3	1.8	2.2	1.6	3.7	1.7	3.3	1.7	3.1
C/C4	1.3	2.4	1.7	4.6	1.3	4.6	1.5	4.9
C/C5	1.2	2.6	1.4	4.5	1.6	5.4		
C/C6	1.7	2.7	1.1	3.6	1.2	3.8	1.2	3.9
C/C7	1.3	2.7	1.6	3.1	1.3	3.5		
C/C8	1.2	2.6	1.7	4.1	1.4	4.4	1.4	4.3
C/C9	1.1	2.6	1.2	3.8	1.2	4.5	1.3	4.2
C/C10	1.1	2.6	1.7	3.0	1.2	3.6	1.2	3.8
C/C11	1.1	2.7	1.1	3.1	1.6	3.0		
C/C12	1.0	2.4	1.3	2.8	1.8	2.8	1.9	3.1
C/C13	1.9	2.4	1.7	2.9			2.0	2.7
Mean	1.4	2.5	1.5	3.6	1.5	3.9	1.6	3.7
S.D.±	0.34	0.27	0.26	0.58	0.26	0.75	0.34	0.75
S.E.±	0.09	0.07	0.07	0.16	0.07	0.02	0.11	0.24

DISCUSSION

The interval of 2.2 ± 0.9 hours, between the feeding of colostrum and the appearance of the labelled IgG, in the plasma (see VI, Results A) is in close agreement with that found by other workers using specific antibodies (Little and Orcutt, 1922, Kerr and Robertson, 1946, McDiarmid, 1946 and Graves, 1963). The same workers obtained a wide range of times for the point at which maximum plasma levels are reached. 5 hours for Brucella agglutinins (Little and Orcutt, 1922 and Graves, 1963), 12 to 16 hours for anti-Trichomonas Foetus antibodies (Kerr and Robertson, 1946) to 24 hours also for Brucella agglutinins (McDiarmid, 1946). By comparison the mean time was 18 ± 5.4 hours in these studies, for IgG_1.

Lack of information about the extravascular distribution of immunoglobulins in the calf has not prevented some workers from attempting to determine relative or absolute absorption efficiencies. Bangham, Ingram, Roy, Shillam and Terry (1958) fed ^{131}I-labelled adult bovine serum and colostral proteins to new-born calves. Although at 3 hours, 8 to 16 % of the labelled proteins were present in the circulation, by 20 hours this had decreased to 5 to 10 %. These workers recognised that their results did not allow for the extravascular distribution

of the labelled proteins. Assuming the plasma volume to be 5% of the body weight, Pierce (1961) estimated that 30% of the Diptheria antitoxin fed to a calf, was present in the plasma 30 hours post partum. This represented a minimum value as no allowance was made for Extravascular loss.

Hammer, Kickhofen and Henning (1968) concluded from their colostral studies that IgM is transmitted less efficiently than γ G. This might indicate some sort of selection between the different immunoglobulin classes. Subsequently, Klaus, Bennet and Jones (1968) claimed that both IgM and IgG were absorbed equally well from the gastro-intestinal tract. Both groups of workers failed to consider the extravascular distribution of these two immunoglobulins. From the initial study in Section V it can be seen that as in other species, IgM is present to a greater extent in the intravascular compartment whereas in Section IV it was found that the opposite was true for IgG.

McEwan (1968) obtained an efficiency of absorption of 46% in 13 bucket fed Ayrshire bull calves, using an E.V/I.V. ratio of 0.65 (Nansen and Nielsen, 1966). The amount of globulin absorbed by the calf was calculated by multiplying the concentration of serum proteins before and after feeding colostrum, by the plasma volume. This quantity of globulin expressed on a body weight basis was then compared with the quantity ingested by the calf, calculated on the same basis.

Subsequently, using the same results and applying the E.V./I.V. ratio of 1.2/1 (calves 1, 6 and 7 in Section IV) McEwan, Fisher and Selman (1970) obtained a value of 65%. This allowed for a 17% casein clot.

Kruse (1970b) in his absorption studies in 141 calves (mostly Black and White Danish or Red Danish) determined an absorption coefficient, assuming a 40% transfer of immunoglobulin to the extravascular pool over the first 24 hours. (This % transfer was calculated from data for human IgG). The highest value he obtained was 43% assuming the plasma volume to be 5% of Body Weight i.e. a maximum of 54% using the mean plasma volume of 63 ml/Kg (Table 3). The mean coefficient or efficiency of absorption for the 72 Black and White Danish calves was 20.1 ± 8.3 (Kruse, 1969).

Of these studies, only the work of McEwan et al (1970) based on globulin levels and the work of Kruse (1970) with "immunoglobulin" levels derived by electrophoresis, have really measured efficiency. However, neither of these methods includes the direct measurement of IgG and both depend on data derived from isotopic studies. Table 19 gives the Efficiency of absorption by both these methods,(Isotopic and Radial Diffusion) both of which are specific for Fast IgG (IgG_1). The measurement of the Efficiency of absorption by both these methods, was the main object of this Section.

These results indicate an Efficiency of 50% (when the mean values of both methods are combined) which is of the same order as the 65% value of McEwan et al (1970) and the maximum corrected value of 54% derived from the results of Kruse (1970).

Although there was a wide scatter in the efficiency with which all 13 calves absorbed colostral IgG (as determined by both methods - see Section VI, Results B and C), the relationship between the amount of IgG (gm) fed and the amount absorbed (see Fig 29) strongly suggests that provided a certain limit (which may vary from calf to calf) is not exceeded, the absorption mechanism, under the varied conditions in these experiments, absorbs a fairly constant proportion of the IgG presented to it - of the order of 50 to 60 %. The upper limit would appear to be reached at around 280gm of IgG in 4 litres of colostrum. Of the five calves offered this quantity, or more, three of them absorbed 23, 27 and 31 % respectively i.e. the efficiency was reduced by about half. Additional work will be required to investigate this limit in relation to the volume of colostrum and the total IgG (gm) fed. If these three calves are also excluded from Table 19, the mean Efficiencies become 52 ± 14 (for the Isotopic method) and 62 ± 7.6 (for the Radial Diffusion method). This would raise the Efficiency to 57% when the results of both methods are combined. Additional evidence concerning the

efficiency of the absorption mechanism comes from comparisons between the isotopic efficiency and the quantity of IgG (gm) fed or absorbed (see Figures 30 and 31). In both graphs, the regression lines, particularly for 10 of the calves (i.e. excluding C/C5, C/C9 and C/C11) are nearly parallel to the abscissa and close to the mean efficiency.

The considerable individual variation in the efficiency of absorption found, may be the result of a number of factors. Firstly, the timing of the feeding of colostrum, i.e. from 3 to 6 hours and secondly, variation in the timing of plasma sample and plasma volume determination over a 24 hour period i.e. 48 to 72 hours. Further, no attempt was made to standardise the quantity of IgG fed, on a body weight basis. In spite of these variations, the basic trend shown by Figures 29 to 31, supports the finding of Selman (1969) that under a particular set of circumstances, a fixed proportion of the immune lactoglobulin presented to a calf may be absorbed. i.e. that Ayrshire calves are born with an equal absorptive capacity.

The distribution of IgG_1, (see Table 22) in the colostrum calves, using both the Sterling and Campbell methods of calculation was similar to that found in the original studies of the metabolism of IgG_1, in Section IV (see Table 2). The measures of catabolism were also similar. When the

apparent distribution was calculated from the colostral data (see Table 21) the mean distribution (E.V./I.V.) for four calves was the same as the Sterling distribution i.e. 1.7/1, but higher than the Campbell distribution of 1.4/1. We have therefore additional evidence that for IgG_1, in the neonatal calf, the extravascular compartment is proportionally larger, relative to the intravascular compartment.

A number of workers have studied the proteinuria associated with the ingestion of colostrum in neonatal calves. Smith and Little (1924) observed that it developed soon after birth and then disappeared after the third day. The low levels and the gradual increase in the protein bound activity found in four calves (see Table 21) in these studies support the finding of Pierce and Johnston (1960) that immune lactoglobulins are largely absent from the proteins (mostly of low molecular weight) associated with the proteinuria.

Other workers may be criticized on the tracers they used for the study of absorption of colostrum. Hardy (1969 a and b) used a high molecular weight PVP (160,000) and an isotopically labelled commercial bovine γ globulin preparation. Finding that after absorption, the plasma activity of both tracers was similar he concluded that the PVP preparation was suitable for absorption studies. In the

first place, although the molecular weights are very similar, the two substances will have very different diffusion coefficients. Thus, the rate and extent to which they equilibrate with the extravascular compartment will be very different. Secondly, the IgG preparation is open to several objections. It will contain a high proportion of IgG_2 which is almost completely absent from colostrum and neonatal calf serum. The method of preparation is such that a probably significant proportion of the protein will be at least partially denatured. In the studies described here, every care was taken to prevent such damage to IgG_1 preparations.

Selman (1969) found evidence of a maternal factor that resulted in very significantly raised immunoglobulin levels in calves left with their dams, although not allowed to suckle them. These calves and the control group were fed from a colostrum pool, on a body weight basis. It is difficult to compare Selmans work with the studies in this Section because of a number of differences with regard to the experimental details e.g. amount and quantity of colostrum, method of feeding etc. However, the Efficiency results in Tables 19 and 20 indicate that a wide range of efficiencies occurred in those calves which were fed and absorbed relatively low quantities of IgG, (i.e. the Group 1 calves, the range being

32% to 81%). Thus, while in no way accounting for the findings of Selman, these results indicate that there is ample room within the range of individual efficiencies for two groups of calves, the immunoglobulin levels of the "mothered" group being 70% higher than the "non-mothered" group. Using the information concerning the distribution of IgG_1, and the isotopic method of measuring Efficiency, it is now possible to investigate more accurately the factors influencing the absorption mechanism itself. While many useful studies have been carried out in large numbers of calves using a variety of estimates of immunoglobulin levels, with the isotopic method for measuring the efficiency of absorption in individual calves, it should be possible to work with much smaller groups of animals e.g. when investigating the mechanism of "shut down" in the neonatal calf i.e. the cessation of the absorption of colostral immunoglobulins.

SUMMARY

The Efficiency with which the new-born calf absorbs Colostral Fast IgG (IgG_1) was investigated by applying the E.V./I.V. ratio of 1.2/1, previously determined (see Section IV). The mean "Isotopic" and "Radial Diffusion" Efficiencies when combined, gave an Efficiency of 50%. When the amount of IgG (gm) fed, was related to the amount absorbed, a fairly constant Efficiency of 50 to 60% was found (up to an apparent limit of 250gm, in 4 litres of colostrum). Further evidence for this was provided by the relationship between the quantities of IgG fed or absorbed and the "Isotopic" Efficiency. Information was obtained about the initial appearance of IgG_1 in the plasma and its relationship to the proteinuria. The distribution of the Colostral IgG_1 over the first 72 hours confirmed the presence of a relatively larger extravascular compartment.

SECTION VII

Plasma Protein Loss via the Gastrointestinal Tract of the Neonatal Calf

INTRODUCTION

Many workers have demonstrated the presence of plasma proteins in the lumen of the alimentary tract. Using autoradiography, Ullberg, Birke, Ewaldsson, Hanson, Uljedahl, Plantin and Wetterfors (1960) found that ^{131}I albumin is discharged into the lumen of the small intestine, in addition to the stomach. The exact role of the gut in normal plasma protein metabolism is however, still debated. On the one hand, Armstrong, Margen and Tarver (1960), Wetterfors, Ullberg, Ulhedahl, Plantin, Birke and Olhagen (1960), Glenert, Jarnum and Riemer (1962), Andersen, Glenert, and Wallervick (1963), Campbell, Cuthbertson, Mackie, McFarlane, Philipson and Sudsaneh (1963) and Wetterfors, (1963), all found evidence that the alimentary tract accounts for a large part, if not most of the albumin catabolised. Their work has been criticised on the grounds that varying degrees of surgery were involved.

Franks, Mosser and Ansladt (1963) concluded that no more than half of albumin catabolism normally occurs in the gut. When they removed most of the jejunum and ileum in rabbits, the catabolism of albumin was not retarded. Jarnum (1963) considered that only 5 to 10 % of albumin catabolism occured in the gut. Freeman (1964) using an improved resin

method (see Jeejeebhoy, 1964) obtained results which supported the view that only a small % of the daily albumin catabolism is due to intestinal leakage.

In pigs, Dich and Nielsen (1964) found that 10 to 30 % of albumin and γ globulin catabolism could be accounted for by intestinal degradation. The same workers (Nielsen and Dich, 1965) found an enormous variation in calves in which albumin and γ globulin were excreted in relatively similar quantities, into the intestine.

Other workers, Rothschild, Oratz and Schreiber, (1966) and Schultze and Heremans (1966) considered the evidence to be very conflicting, a view which had previously been expressed by Freeman (1964). Waldmann (1966) felt that further work was necessary before the exact magnitude of protein gut loss in normal subjects is understood and the mechanism elucidated.

Rothschild, Oratz and Schreiber (1966) and (1970), Freeman (1967) and McFarlane (1969) all considered that catabolism occurred throughout the body, many tissues possessing the potential, the exact fraction lost in the gut being either unknown or small.

In addition to the normal leakage of plasma protein into the alimentary tract, it has been known for some time that certain diseases are associated with an abnormally

large loss of plasma proteins into the gut. The discovery of these protein-losing gastroenteropathies (a term introduced in an Editorial in the Lancet, 1959), was largely due to the observation that ^{131}I labelled plasma proteins given intravenously were rapidly excreted in the measurable quantities into the alimentary tract of patients with certain diseases, but not to any extent in normal people. The association between hypoproteinaemia and protein loss into the alimentary tract was investigated by Steinfeld, Davidson and Gordon (1957), Holman, Nickel and Sleisenger (1959), Steinfeld, Davidson, Gordon and Greene (1960), Jarnum (1963), Jeejeebhoy (1964) and Jarnum, Jensen and Bro-Jorgensen (1966). Waldmann and Schwab concluded that it was a bulk loss of all plasma proteins associated with the hypercatabolism of albumin and IgG. Waldmann (1966) in his review, lists over forty gastrointestinal conditions in which excessive loss of plasma proteins has been demonstrated.

Hypercatabolism associated with gut loss of plasma proteins has been demonstrated in a number of animal diseases. Nielsen (1966a) investigated Ostertagiasis, Johnes Disease and chronic catarrhal abomasoenteritis in the bovine, terminal ileitis (Nielsen, 1966b) and chronic enterocolitis (Nansen and Nielsen, 1967) in the pig, both being associated with

diarrhoea.

Marsh, Mebus and Underdahl (1969) found a significantly increased loss of all serum and unabsorbed milk proteins via the intestinal tract in diarrhoeic neonatal calves. They concluded that the observed loss of proteins via the faeces during diarrhoea could seriously reduce the chances of the calf surviving severe diarrhoea or complicating secondary infections.

Subsequently, de la Fuente (1970) showed a close correlation between immunoglobulin levels in calves and their subsequent ability to survive an attack of neonatal diarrhoea.

The preparation of ^{131}I labelled Polyvinylpyrrolidone (PVP) by Gordon (1959) marked the beginning of a new era in the investigation of alimentary diseases. Its' application was discussed by Holman, Nickel and Sleisenger (1959). Steinfeld, Davidson, Gordon and Greene (1960) found a raised PVP faecal activity in patients with active regional enteritis or ulcerative colitis. Jarnum (1961) considered that an abnormal PVP test was the most reliable indication of a gastro-intestinal leak and the only means of estimating the size of the leak. Jeffries, Holman and Sleisenger (1962) in their review of the relationship between the plasma proteins and the alimentary tract considered the use of labelled PVP

to be of proven qualitative value, although limited by a number of factors. van Tongeren and Majoor (1966) stated that it gave an approximate estimate of the loss of protein through the intestinal wall. When given orally only 50% to 90% was recovered, due either to absorption or breakdown. Waldmann (1966) was of a similar opinion, Dargie (1969) found a good correlation between the faecal excretion of PVP and the inferred increased plasma protein losses.

Thus, bearing in mind the quantitative limitations of the method, it was decided to carry out an initial trial with ^{131}I PVP in two diarrhoic calves and the two older control calves.

The labelling of plasma proteins with $^{51}CrCl_3$ was first described by Gray and Sterling (1950). Waldmann (1961) and Waldmann and Wocher (1963) considered that the label was of considerable value in the study of enteric protein loss. They recovered 93 to 98 % of the label in the faeces following oral administration, and obtained a direct correlation between ^{51}Cr faecal excretion and the fractional catabolic rate of ^{131}I albumin. These findings were confirmed by Guillen and Peterson (1964). van Tongeren and Majoor (1966) showed that there was no advantage in in vitro labelling of albumin with $^{51}CrCl_3$ as against in vivo labelling. van Tongeren and

Reichert (1966) obtained a normal mean clearance of 13 ml/day whereas patients with protein-losing gastroenteropathy had much larger clearances of up to 600 ml/hour. (similar results had been obtained by Waldmann and Wochner, 1963). The technique has been further investigated, discussed and applied by many other workers to the study of protein-losing gastroenteropathies.

Other labels, ^{95}Nb labelled albumin and ^{67}Cu labelled ceruloplasmin have been used (see Dargie, 1969). ^{59}Fe labelled Dextran was described by Anderson and Jarnum, (1966), Jarnum, Westergaard, Yssing and Jensen (1968). It gave similar results to the $^{51}CrCl_3$, and is not readily available.

Thus, to quantitate the loss of IgG and IgM in the alimentary tract in calf scours it would be necessary to use $^{51}CrCl_3$ simultaneously with ^{125}I labelled immunoglobulins and try and relate the increased excretion of the ^{51}Cr label in the faeces with increased immunoglobulin metabolism.

MATERIALS AND METHODS

Three separate experiments were carried out. An initial trial with ^{131}I - PVP in four calves, two older control calves and two diarrhoeic calves. A comparison was then made between the % recovery of three isotopic tracers given orally, ^{131}I - PVP (40,000 av. Mol,wt.), ^{125}I - PVP (160,000 av. Mol.wt.) and $^{51}CrCl_3$. Finally $^{51}CrCl_3$ was used in five non-diarrhoeic calves, to measure the leak of plasma proteins into the alimentary tract.

The presence or absence of diarrhoea was determined by a daily visual assessment of the faeces. Calves were considered to be diarrhoeic when they produced faeces corresponding to the ++ and/or +++ classification of de la Fuente, 1970.

A. Faecal Excretion of ^{131}I - PVP in Diarrhoeic and Control Calves

1. Source of Calves

Calves 15 and 16 were purchased from Mr A. Guthrie, Moss Side Farm, Kilmarnock when 2 to 5 days old. Calves 9 and 13 had been previously obtained from the same source and were now 12 and 15 weeks old respectively.

2. Injection

Each calf received a known quantity of ^{131}I - PVP (1.7 to 3.1 mc, roughly in proportion to body weight) by intravenous injection. The Plasma Volume was determined with ^{125}I labelled IgG_1.

3. Collection of Urine and Faeces

The calves were kept in tubular cages (see Section II.A.2). A Folatex 60 balloon catheter (size 12 FG - 5 to 15 cc, from Vicarey, Davidson and Co., Glasgow) was inserted into the bladder of C 9, a heifer calf, and secured by folding a strip of elastoplast round it, leaving a flap which was then sutured under local anaethesia to the skin. A polythene tube was then attached to the catheter. The faeces were collected on the grid and the tray beneath. To collect the urine from C 13 the top was removed from a disposable urine

Table 26

Daily Faecal Clearance of ^{131}I-PVP in Diarrhoeic and Control Calves (ml)

	Control Calves		Diarrhoeic Calves	
Day	No.9	No.13	No.15	No.16
3	50	14.9	111	202
4	77	46	65	
5	78	12.3	181	56
6		39	died	died
Mean	68	28	119	86
S.D.±	15.7	16.9	58	104
S.E.±	9.1	8.5	33	60

drainage bag (Portland Plastics Ltd., Hythe, Kent), the cut edge reinforced with elastoplast and then sutured under local anaesthesia round the end of the prepuce. A polythene tube was then attached to the outlet. The faeces of C 13 were collected as for C 9. The urine and faeces of Calves 15 and 16 were collected by the method of de la Fuente (1970) - see Section II A 2b.

4. Further Experimental Details

See Section II.

5. Calculation of Results

Cummulative faecal output (%) and Faecal clearance (ml per day) were calculated (see Section II). To enable valid comparisons to be made between the two groups, it was necessary to correct the cummulative activity for the control calves to a 3 day period and both measures of faecal excretion were adjusted for the differences in body weight and plasma volume, between the calves.

6. Results

The results are shown in Tables 26, 27 and 28 and the plasma disappearance curves of one control and one diarrhoeic calf are shown in figure 32. Although the apparent plasma $T\frac{1}{2}$ for both diarrhoeic calves was shorter than the $T\frac{1}{2}$ of the

Table 27

Faecal Excretion of ^{131}I-PVP in Diarrhoeic and Control Calves

	Calf No.	Clearance ml	Clearance ml/Kg	Clearance % P.V.	Excreted %	Excreted %/Kg	Excreted %/P.V. (litres)
CONTROL	9	68	1.1	1.8	0.96	0.015	0.250
	13	28	0.7	1.1	0.51	0.013	0.197
	Mean	48	0.90	1.5	0.73	0.014	0.223
	S.D.	28	0.28	0.5	0.31	0.001	0.037
DIARROEIC	15	119	7.9	7.8	2.6	0.093	1.71
	16	86	6.7	4.1	1.9	0.063	0.913
	Mean	103	7.3	6.0	2.3	0.078	1.31
	S.D.	23	0.84	2.6	0.50	0.021	0.56
"t" Test		N.S.		p 0.01	N.S.		p 0.05	N.S.

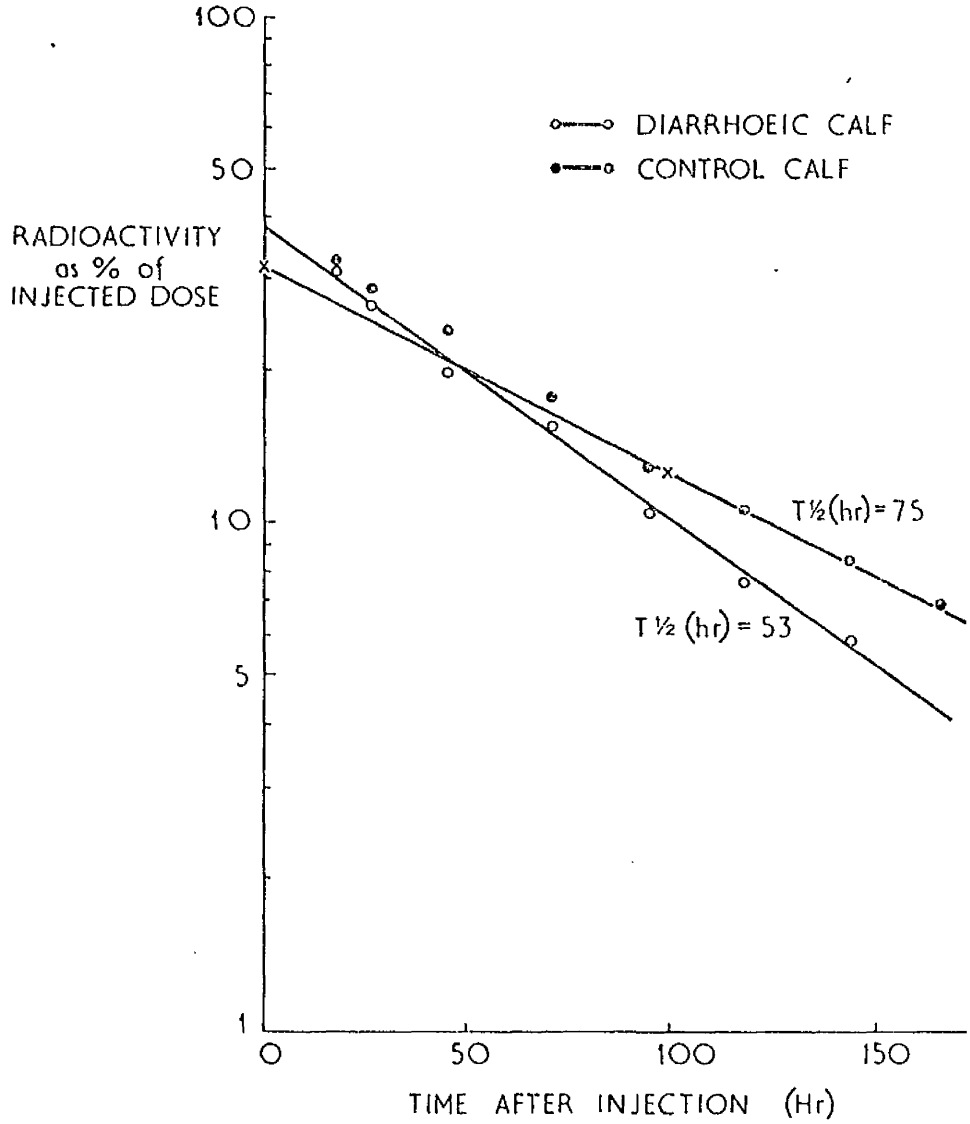
Table 28

Additional Data for Calves in Tables 26 and 27

Calf No.	Total Albumin (gm/100ml)	Total Globulin (gm/100ml)	Mean P.C.V.	PVP $T_{\frac{1}{2}}$ (hours)	Injected Activity (mc)	P.V. (ml/Kg)
9	2.2	3.3	31	75	3.1	59
13	1.9	3.5	28	66	2.9	65
15	1.3	3.6	44	63	1.7	53
16	1.6	3.6	45	53	1.8	69
't' Test -	-	-		N.S.	-	-

Figure 32

PLASMA DISAPPEARANCE of ^{131}I - PVP (40,000 av. Mol. wt) in a DIARRHOEIC and a CONTROL CALF



control calves, the difference was not significant. Because of the differences in Body Weight and Plasma Volume between the calves, the increases found in both measures of Faecal Excretion of ^{131}I - PVP were not marked. A greater increase noted in the % of the label excreted in the faeces, over 3 days, than in the mean daily clearance over the same period (see Table 27).

When the results were expressed as a fraction of body weight, the faecal plasma clearance of 7.3 ± 0.84 ml/Kg/day in the diarrhoeic calves was very significantly larger than the control level of 0.90 ± 0.28 ml/Kg/day. Similarly the % excreted activity was 0.078 ± 0.021 %/Kg compared to 0.014 ± 0.001 %/Kg for the control calves. The same measures when expressed as fractions of the plasma volume showed similarly elevated levels, which were not however statistically significant, the two groups being very small.

The results clearly demonstrate an increased loss of macromolecules into the gut of the diarrhoeic calves.

Table 29

Comparison between the Faecal Recovery (%) of ^{131}I-PVP(40,000 av. Mol.wt.), ^{125}I-PVP(160,000 av. Mol.wt.) and $^{51}CrCl_3$, over 3 days.

Calf no.	^{131}I-PVP	^{125}I-PVP	$^{51}CrCl_3$
1	46	62	
2	71	66	
3	36	30	
4		75	72
5		61	117
6		45	136
Mean	51	57	108
S.D.±	18	16	33
S.E.±	10.4	6.6	19

B. Faecal Recovery of ^{131}I PVP (40,000 av. Mol. wt.), ^{125}I PVP (160,000 av. Mol. wt.) and $^{51}CrCl_3$, given orally.

1. Source of Calves

Six market calves were purchased through a dealer. They were up to one week old.

2. Administration

See Section II C 4.

Three calves received 100μc of $^{51}CrCl_3$ and 100μc of ^{125}I PVP. The other three received 150μc of ^{131}I PVP and 100μc of ^{125}I PVP. All three isotopes had been diluted with saline so that each calf received approximately 10 ml of each label, in a weighed syringe.

3. Samples

Faeces were collected for 3 days following dosing, using long polythene bags attached to the calves (see Section II A 2b). All of the calves had developed diarrhoea, the severity of which varied considerably. The total amount of each label in the faeces was determined and expressed as a % of the quantity administered.

Results

The results are shown in Table 29. It can be seen from the mean recoveries of 51 ± 18 % and 57 ± 16 % for the

two PVP labels that there is little to choose between them. The mean recovery of the $^{51}CrCl_3$ label on the other hand was 108 ± 33 %.

Table 30

Repeat Determinationsof the % Faecal Recovery

of ^{125}I-PVP (160,000 av.Mol.wt.) and $^{51}CrCl_3$, over 3 days

Calf No.	^{125}I-PVP	$^{51}CrCl_3$
2	42	125
3	35	96
4		125
5	37	89*
6	44	114
Mean	40	110
S.D.±	4.2	17
S.E.±	2.1	7.4

* Incomplete collection of faeces

C. Repeat determination of the Faecal Recovery of ^{125}I PVP and $^{51}CrCl_3$

1. Calves

Five of the calves from the initial experiment were used (see above).

2. Other Experimental Details

See VII B above.

Four calves received both labels, but the remaining calf was only given $^{51}CrCl_3$. The interval between the experiments was one week. With the exception of Calf 5 which was still scouring profusely, the calves were no longer diarrhoeic.

Results

The results are shown in Table 30.

110 ± 17 % of the $^{51}CrCl_3$ was recovered but only 40 ± 4.2 % of the PVP. Although this recovery of PVP is lower than in the first experiment, the difference is not statistically significant. From the results of both these oral dosing experiments, it can be seen that whereas approximately 100% of the $^{51}CrCl_3$ label is recovered only 40 to 50 % of either of the labelled PVP's is present in the faeces. The rest

of the PVP will have broken down in the gut or been absorbed. Thus for the quantitation of plasma protein loss into the gut of the neonatal calf, the use of $^{51}CrCl_3$ is indicated. Similar recoveries have been obtained in man and other species.

The fact that in most of the calves the recovery of $^{51}CrCl_3$ was more than 100%, emphasised the problems associated with obtaining a suitable aliquot of calf faeces for "counting". In both these experiments the problem was increased by the bulking of 3 days faeces. The level of activity in the faeces will have varied considerably over this period.

D. Plasma loss in Non-diarrhoeic Calves measured with $^{51}CrCl_3$

1. Source of Calves

Calves Cr/1 to Cr/4 were retained from the faecal recovery experiments. They were by this time approximately 3 weeks old and no longer diarrhoeic. C/C 10 was one of the calves used in Section V which had shown no evidence of diarrhoea since birth. It was also 3 weeks old.

2. Injection

Each calf was given a known quantity of $^{51}CrCl_3$ by intravenous injection. At the same time, the plasma volume was measured with Evans Blue, see Section II F, except for C/C 10. In this case, the plasma volume was measured with ^{125}I labelled Fast IgG.

3. Collection of Faeces

See Section II A 2b.

4. Further experimental details

See Section II.

5. Calculation of Results

Cumulative faecal output (%) and faecal clearance (ml/day) were calculated.

Table 31

Daily Faecal Clearance of $^{51}CrCl_3$ in Non-Diarrhoeic Calves (ml)

Day	Cr/I	Cr/2	Cr/3	Cr/4	C/C10
3					44
4	105	59			60
5	69	48	18	27	30
6	50	75	57	44	36
7	74	49	25	15	31
8		65			
Mean	74	59	33	29	40
S.D.±	22.8	11.3	20.8	14.6	12.4
S.E.±	11.4	5.1	12.0	8.4	5.5

Table 32

Faecal Excretion of $^{51}CrCl_3$ in Non-Diarrhoeic Calves

Calf No.	Mean daily Clearance(ml)	Cumulative Faecal Output(%)	Apparent Plasma $T_{\frac{1}{2}}$
Cr/1	74	3.4	4.9
Cr/2	59	1.8	5.1
Cr/3	33	1.8	5.1
Cr/4	29	0.80	5.5
C/C10	40	1.4	6.0
Mean	47	1.8	5.3
S.D.±	19	0.96	0.43
S.E.±	8.5	0.42	0.17

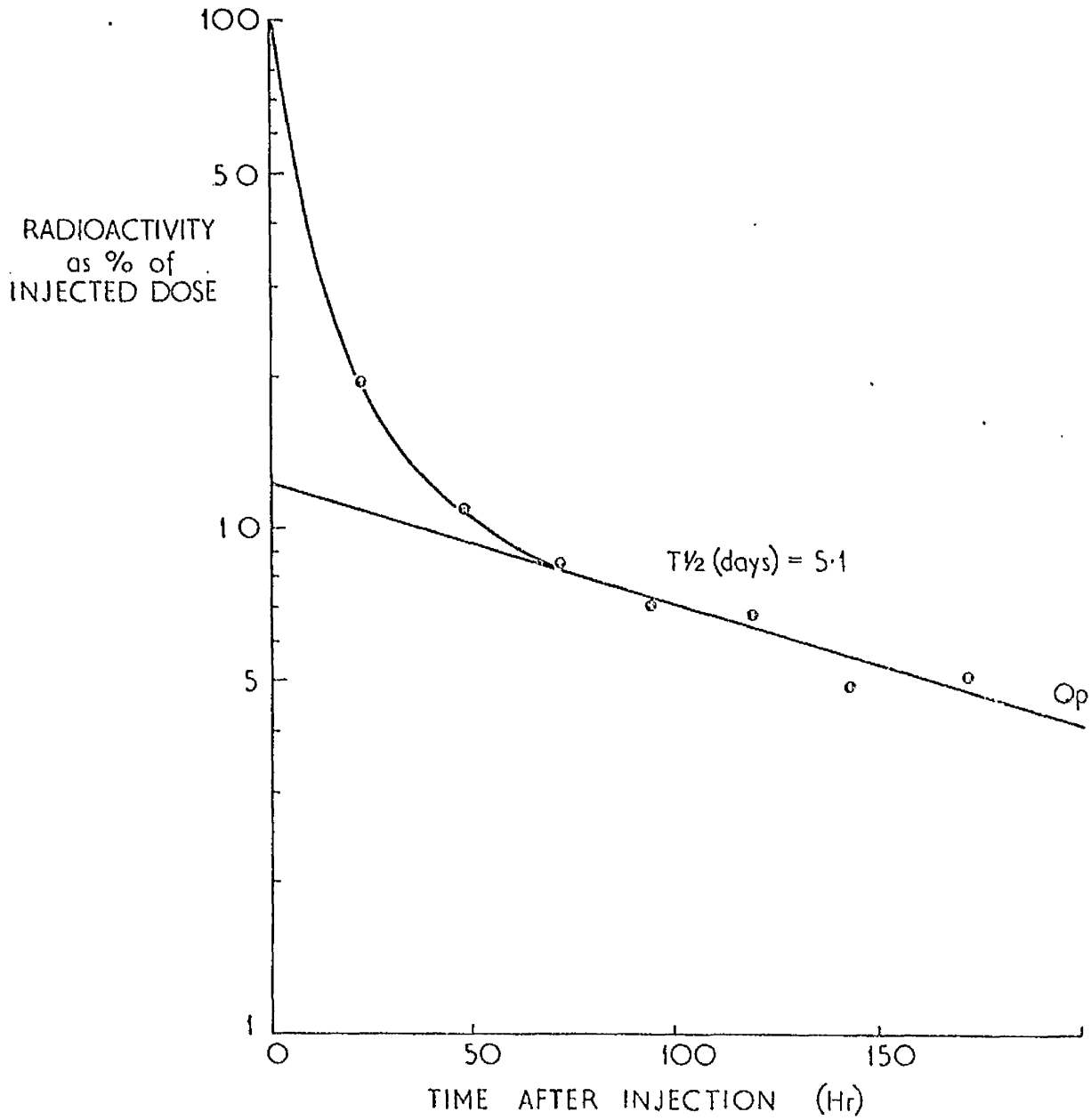
Table 33

Additional Data for Calves in Tables 31 and 32

Calf No.	Total Albumin (gm/100ml)	Total Globulin (gm/100ml)	P.V. (ml/Kg)	Injected Activity (μc)
Cr/1	1.8	4.9	54	500
Cr/2	1.6	4.5	49	500
Cr/3	1.7	4.0	55	500
Cr/4	1.6	4.1	54	500
C/C10	1.2	3.7	63	800
Mean	1.6	4.2	55	
S.D.±	0.22	0.46	5.0	
S.E.±	0.10	0.20	2.3	

Figure 33

DISAPPEARANCE of $^{51}CrCl_3$ in a NON-DIARRHOEIC CALF (Cr/3)



Results

The results are shown in Tables 31, 32 and 33 and the plasma disappearance of the activity in one of the calves, in Fig. 33 . The mean $T_{\frac{1}{2}}$ for the group was 5.3 ± 0.43 days. A mean daily clearance of 47 ± 19 ml was found, which represented 2.2 ± 0.98 % of the plasma volume/day. 1.8 ± 0.96 % of the injected activity was recovered in the faeces over a 3 day period.

The control ^{131}I PVP clearance as a % of P.V. of 1.5 ± 0.5 % (see Table 27), previously obtained, thus represents about 2/3 of the $^{51}CrCl_3$ clearance, similarly expressed. A difference of this order would have been expected in view of the lower recovery of orally administered labelled PVP. (See Section VII, B and C).

DISCUSSION

On the basis of the 6% of the intravascular pool of IgG catabolised per day (see Section IV D - Results) the 2.2 ± 0.98 % of the plasma volume per day, can only account for 1/3 to 1/2 of the IgG catabolised. As the calves used for the $^{51}CrCl_3$ studies were 3 to 4 weeks old, their catabolic rate would have been higher than 6%. Thus for Fast IgG, it would appear that the gut of the neonatal calf does not account for most of the catabolism, although making a significant contribution.

Exactly how a plasma protein loss of 100 ml or more per day (see Table 27) occurs in diarrhoeic calves is not known. Jarnum (1963) considered that there were three possible mechanisms for the normal loss into the gut, secretion, in extracted epithetial cells or by a passive leak. Where excessive loss occurs in the presence of abnormal epithelium, he considered it to be due to abnormally high capillary permeability. Schwartz (1963) suggested that such losses occur between the cells. Bierring, Jarnum and Schwartz (1965) using the electron microscope found that the intercellular spaces may become markedly dilated, containing a precipitated flocular material. Jarret, Miller and Murray (1970) showed, in addition to this

leak between the cells, a marked leak from the small blood vessels of the villi, in their study of the response to Nippostrongylus infection in rats. The state of the intercellular junctions of the gut epithelium in the diarrhoeic calf has still to be investigated.

While only a little is known about the mechanism of macromolecular leaks into the gut, it has been known for a long time that neonatal diarrhoea in calves, as in other species, is usually associated with E. coli.

Since the early work of Jensen (1893), this relationship has been investigated and discussed by many workers. Wood (1955) found that calves which had had colostrum died of a localised intestinal infection and not from coli-septicaemia. Dunne, Glantz, Hokanson and Bortree (1955) claimed to reproduce the disease experimentally and prevent it with bacteriostatic agents. They emphasised the apparent spontaneous development of outbreaks. Smith (1962) found no excessive proliferation of the organism, failed to transmit the disease and concluded that it was probably not infectious. Attempts to prevent colibaccillosis by vaccination were apparently unsuccessful (Gay, McKay and Barnum, 1964 a, b, and c). Loosmore, Anderson and Edgson (1964) described environmental build up. Roy (1964) while recognising doubt about the cause of death in

enteric colibaccillosis, concluded that E. coli may be of considerable importance.

Gay (1965) regarded it as only part of the syndrome of the scouring calf. He and Sojka (1965) considered that there was some evidence that certain strains could produce scouring in calves and that specific antibody might give protection. Sojka while stating the general consensus that E. coli is the organism most commonly isolated, shared the opinion of Gay that almost nothing is known about the aetiology of the condition, earlier work having only emphasised its complexity. Penhale (1965) was unable to demonstrate an unequivocal relationship between scouring and the administration of experimental serotypes. Sojka arrived at a similar conclusion, on the available evidence.

Amstutz (1965) in a symposium on calf diarrhoea considered that infectious diarrhoea was the main cause of the high neonatal calf mortality. Another contributor, Radostits (1965) considered the role of E. coli obscure and a causal relationship difficult to establish. Smith and Halls (1967 a and b) studied the possible role of E. coli enterotoxin. The endotoxin had been fully investigated by Penhale (1965). De la Fuente (1970) found considerable variation in the severity and duration of neonatal diarrhoea in calves. He concluded that a high concentration of antibody would give

protection, over and above all other factors, including treatment. However, Smith (1971) concluded from bacteriological studies that neonatal diarrhoea is generally non-infectious in origin.

While most workers accept that E. coli plays an important role in neonatal diarrhoea, other agents have been discovered and investigated. Baker (1943) and Brandly and McClurkin (1956) described a virus enteritis, accompanied by pneumonia. The possible role of viruses was discussed by de la Fuente (1970). Mebus, Kono, Underdahl and Wiehaus (1971) described experimental virus diarrhoea in neonatal calves. The presence or absence of viral agents in most outbreaks of "calf scours" has however yet to be demonstrated.

In relation to neonatal calf diarrhoea, a number of points have been established in these studies, or are widely accepted. These are, the hypercatabolism of Fast IgG (see Section IV), a macromolecular leak (using ^{131}I PVP, 40,000Mol.wt.) and the association with an enteric form of Coli-baccillosis.

The most likely cause of the hypercatabolism of IgG is the increased plasma leak. Whether it is sufficient to account for the total increase has yet to be investigated. There may be an associated increase in endogenous catabolism as well. Also unknown is whether this loss of plasma into

the gut is a bulk process, unrelated to protein size. In man, this would generally appear to be the case (Strober, Blaese and Waldmann, 1970), the relative increases in the Fractional Catabolic Rates of different plasma proteins being similar. However in Crohns Disease, the increase in the catabolism of IgG is 50% higher than the albumin increase. (Jarnum, Bendixen, Jensen, Solloft, Weeke and Westergaard, 1970). Further investigation is therefore required.

Whether or not there is a preferential leak of a particular immunoglobulin into the gut in this enteric disease, the presence of immunoglobulins in the alimentary tract may influence the course of the disease. The presence of antibody in the faeces (copro-antibody) was first described by Davies (1922). Gillen, Young, Massey and Brande (1960) and Freter and Gangarosa (1963) found that circulating antibody had little effect on enteric infections. The latter workers found that oral vaccination was the only method of maintaining coproantibody against cholera. Hooper and Haelterman (1966) showed that the presence of antibody in the lumen of the gut was necessary to protect pigs against Transmissible Gastroenteritis. This was later confirmed by Cartwright (1969).

Schultze and Heremans (1966) concluded that coproantibody was locally produced IgA and that it performed a protective

role in the defence of the gut mucosa. Felsenfeld (1968) confirmed earlier doubts about the unreliability of serum antibody levels with regard to the actual immune status against enteric agents and the importance of IgA in man. According to Jarnum et al (1970), 90% of this IgA is produced locally in the gut.

There is conflicting evidence for the importance of specific antibody in protecting calves against the enteric form of coli-baccillosis. McEwan (1950) considered immunity to it, to be independant of specific antibody. Spain, Bradess and Greenblatt (1956) concluded that γ globulin levels were not the determining factor with regard to death from neonatal diarrhoea, although an immuno mechanism might also be involved. Penhale (1965) showed that failure to demonstrate antibodies could often be accounted for in terms of the technique used to detect them. Thus earlier negative results, did not necessarily reflect the true position. More recently de la Fuente (1970) found that the calves which survived an attack of Neonatal Diarrhoea were those with the highest immunoglobulin levels, as measured with the Zinc Sulphate turbidity test. However, Harwell and Fey (1970) were unable to show any correlation between low γ globulin levels in market calves and diarrhoea. de la Fuente (1970) suggested that there must be an immunological

mechanism, sufficient to neutralise the bacteria and their products, with respect to diarrhoea. Such a mechanism might be accounted for simply in terms of a bulk loss of all plasma proteins into the gut, including immunoglobulins, or there may be an additional preferential secretion. This will have to be differentiated from increased endogenous catabolism, simply associated with a bulk loss.

Kramer (1963) and Penhale (1965) demonstrated that in the bovine, as in other species, (Gitlin, Rosen and Michael, 1963, Michael and Rosen, 1963, Robbins, Kenny and Suter, 1965 and Hanson and Winberg, 1966), the main antibodies to E.coli are IgM immunoglobulins. A solution to the problem of the presence or absence of a preferential leak of one or more immunoglobulins must await a satisfactory IgM preparation (see Section V) suitable for metabolic studies. While there is little or no direct evidence at the moment to suggest that IgA may also be important in this respect, its' relationship to alimentary disease in other species (see above) suggests that it should also be investigated. It will thus be necessary to see if the metabolism of one or all three immunoglobulins is influenced to a greater extent by diarrhoea than the metabolism of albumin (hypercatabolism of Fast IgG has already been demonstrated - see Section IV).

SUMMARY

A significantly increased leak of macromolecules into the alimentary tract of neonatal diarrhoeic calves was demonstrated with ^{131}I-labelled PVP. Subsequently, this tracer was shown to under estimate the plasma leak. $^{51}CrCl_3$ was found to be a more suitable label for quantitative studies. In an initial study in non-diarrhoeic calves, a plasma loss of approximately 50 ml/day was demonstrated. This would account for less than half of the IgG_1 catabolised/day.

SECTION VIII

GENERAL DISCUSSION

The work described in this thesis represents the first direct measurement of the Efficiency of Absorption of Colostral Fast IgG (IgG_1) in the Neonatal Calf. Before the colostral studies could be carried out, information was required about the distribution and catabolism of IgG_1. This was obtained from a study of the metabolism of IgG_1, using a purified immunoglobulin preparation, trace labelled with the isotopes of iodine.

It had been intended to carry out a similar investigation into the absorption of Colostral IgM. However, the work with IgM (see Section V) failed to produce undenatured IgM preparation, suitable for metabolic and colostral studies. This work will require further study (see Discussion - Section V).

When the IgG_1 metabolic studies started, there was considerable evidence for the existence of both the Bovine IgG subclasses, termed Fast and Slow or IgG_1 and IgG_2 (and for a Bovine IgM). At a Symposium on Bovine Immune System (Butler, Winter and Wagner, 1971), it was proposed that these subclasses should be known as IgG_1 and IgG_2. Both nomenclatures have been used in this thesis. The existence of a Bovine IgA class, reported by Mach, Pahud and Isliker (1969), was also recognised. The same workers (Mach and Pahud, 1971) showed that it was homologous to Human IgA. In the future, a study of the metabolism and absorption of Colostral IgA will have to be made.

Evidence was found (Section VII A) of an increased macromolecular leak of plasma proteins into the alimentary tract of the diarrhoeic calf. Such a loss may considerably reduce the immunoglobulin status of the calf. Assuming that this diarrhoea represents an enteric form of Colibacillosis (see Section VII) Discussion), IgM antibodies will be important. Porter and Noakes (1970) showed that IgA is the major intestinal immunoglobulin in the 5 week old calf. Thus the metabolism of both these immunoglobulins will have to be studied to show if the process is one of equal leakage of all plasma components, or whether some or all of the immunoglobulins are preferentially "leaked" into the alimentary tract. Hypercatabolism of IgG_1 has already been demonstrated (Section IV D).

The mechanism by which the maternal immunoglobulins are absorbed by the calf is not fully understood. The possible role of hormonal or other humoral factors in the regulation of absorption, has been suggested but requires further study (Butler et al, 1971). The first stage for such a study, the direct measurement of the Efficiency of Absorption has, as already indicated, been carried out for IgG_1. The absorption of IgM and IgG will have to be similarly quantitated before factors effecting the absorption of all these immunoglobulins can be fully investigated. At the moment, this is only

possible for IgG_1.

No evidence was found in the absorption efficiency studies (Section VI) of premature "shut down" (i.e. an early loss of absorbing capacity). This is in keeping with the findings of Kruse (1969) and Selman (1969). However, Kruse (1970c) showed that under normal farming conditions, a certain level of hypogammaglobulinaemia is inevitable. Further studies are required to find the best way to enable calves to make up this deficiency. When more is known about the factors involved in "shut down", it may be possible to delay or even temporarily reverse the process. Alternatively, once the relative importance of the immunoglobulins has been fully worked out, Immunoglobulin preparations or enriched fractions might be used as parenterally administered colostrum substitutes. Some work along these lines has already been carried out (Logan and Penhale, 1971). Similar preparations might also be used in the prophylaxis and treatment of Neonatal Diarrhoea.

The investigation of factors involved in the absorption of IgG_1, is now possible. Further work will be necessary before similar studies can be carried out for IgA and IgM. It will then be possible to investigate the role of all three immunoglobulins in Neonatal Diarrhoea.

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THE METABOLISM OF PLASMA PROTEINS IN THE YOUNG CALF

Summary of a thesis submitted for the degree of
Doctor of Philosophy of the University of Glasgow
by Dugald F. Macdougall, B.V.M.S., M.R.C.V.S.

The work described in this thesis represents the application of isotopic tracer techniques to a study of the metabolism of the immunoglobulins absorbed from colostrum by the new-born calf. While previous studies, based on serum immunoglobulin levels, have greatly added to our knowledge of this absorption, for precise quantitation it is necessary to know how the immunoglobulins are distributed between the intravascular and extravascular compartments, the rates of equilibration between the compartments and the rates of catabolism, in the neonatal calf. These measurements can only be made satisfactorily using isotopically labelled proteins.

Bovine Fast IgG (IgG_1) was prepared from neonatal calf serum or colostral whey by a combination of molecular sieve and ion-exchange chromatography, and trace labelled with radio-iodine. The metabolism of IgG_1 was then studied in Ayrshire calves, less than 1 week old. A distribution of 1.2/1 (Extravascular/Intravascular) was obtained, equilibration being complete by 48 to 72 hours. The occurrence of diarrhoea in some of the calves had a marked effect on the catabolic

rates but not on the distribution, although a close correlation was found between the distribution of IgG_1 and all four measures of catabolism. The relative size of the extravascular compartment decreased with age. The albumin distribution was found to be 1.9/1 (Extravascular/Intravascular).

Bovine IgM was prepared from colostral whey by molecular sieve chromatography and similarly labelled. In metabolic studies four separate preparations all showed evidence of denaturation and were thus not suitable for providing the information required for absorption studies. Denaturation criteria were investigated. The distribution of two of the preparations indicated that IgM (as in other species) is retained to a greater extent within the circulation, than IgG.

Using the information obtained from the metabolic studies, it was then possible to quantitate the efficiency with which the new-born calf absorbs colostral IgG_1. Colostrum containing a tracer quantity of ^{131}I labelled IgG_1 was fed by stomach tube to Ayrshire calves, 3 to 6 hours post partum. 48 to 72 hours later, the plasma volume was determined with ^{125}I labelled IgG_1. The total absorbed activity was then calculated and expressed as a % of the activity in the colostrum. IgG levels were also directly measured and the efficiency similarly calculated. When the mean results from both methods were combined, an overall Efficiency of 50% was obtained. The close correlation between

the amount of IgG (gm) fed and the amount absorbed indicated a similar efficiency (up to a limit of 250 gm, in 4 litres of colostrum). Information was also obtained about the initial appearance of IgG_1 in the plasma and its relationship to the proteinuria.

A significantly increased leak of macromolecules into the alimentary tract of neonatal diarrhoeic calves was demonstrated with ^{131}I-PVP (40,000 av. Mol. wt.). This tracer was subsequently shown to be under-estimating the plasma leak and $^{51}CrCl_3$ was found to be more suitable as a label for quantitative studies. Using $^{51}CrCl_3$, a plasma loss of approximately 50 ml/day was demonstrated in non-diarrhoeic calves which would account for less than half of the IgG_1 catabolised / day.

The investigation of factors influencing the absorption of IgG_1 by the new-born calf, is now possible. Similar IgM studies will have to wait until the denaturation problems can be overcome. In addition, a Bovine IgA is now recognised and will require study. It will eventually be possible to investigate the role of all three immunoglobulins in relation to Neonatal Diarrhoea.

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